# THESIS

**In order to obtain**

## DOCTORATE OF UNIVERSITÉ DE TOULOUSE

**Issued by** Université Toulouse III – Paul Sabatier
**Speciality:** Pharmacology

---

**Presented by:**
**Arba Pramundita Ramadani**

**Title:**
Various Antimalarial Strategies in Indonesia to Fight *Plasmodium falciparum*

---

**JURY**

| | | |
|---|---|---|
| Françoise BENOIT-VICAL | Research Director INSERM, Toulouse | Thesis Promotor |
| Mustofa | University Professor, Indonesia | Thesis Co-Promotor |
| Alexis VALENTIN | University Professor – Hospital Practitioner, Toulouse | Examiner |
| Mahardika Agus Wijayanti | Research Director Faculty of Medicine, UGM, Indonesia | Examiner |
| Supargiyono | University Professor, Indonesia | Reviewer |
| Iwan Dwiprahasto | University Professor, Indonesia | Reviewer |
| Sofia Mubarika Harjana | University Professor, Indonesia | Reviewer |

---

**Doctoral Program** Biologie – Santé – Biotechnologie
**Research Unit** LCC-CNRS, UPR8241 Toulouse, France
**Thesis Promotors** Françoise BENOIT-VICAL and Mustofa

# ACKNOWLEDGMENT

Owing to Allah the Almighty mercy and blessings, I could finally complete this dissertation entitled "Various antimalarial strategies to fight *Plasmodium falciparum* in Indonesia". This means my long full-of-romance journey at Université Toulouse III - Paul Sabatier and Gadjah Mada University is over. Having completed this study, I hope to get the ease and ways to learn from this journey, so I can dedicate the rest of my life to the benefit of my family, institution, and community through the will of Allah the Almighty.

First of all, I would like to thank Dr. Azzedine BOUSSEKSOU for accepting me into the research group in LCC-CNRS and Prof. Nicolas FABRE for allowing me to conduct the research in Pharmadev.

I am deeply indebted to **Dr. Françoise Benoit-VICAL** as my supervisor who has kept on guiding me as her “student daughter” from the very beginning until the end of my writing with her patience and wisdom. I learn a lot from her scientific ways of thinking, the exchanges of ideas and issues, immense knowledge and lot other things.

Grateful acknowledgement is also made to **Prof. Dr. Mustofa, M.Kes. Apt**, my Co-Supervisor who provided me an opportunity and continuous support during my study and related research. His patience and guidance helped me in all the time of research and writing this dissertation. His wisdom teaches me a lesson never being taught in class or written in a book.

A profound gratitude to the members of my dissertation defence committee: Prof. Alexis VALENTIN and Dr. dr. Mahardika Agus Wijayanti, DTM&H, M.Kes. as the examiner; also Prof. Iwan Dwiprahasto, Prof. Supargiyono and Prof. Sofia Mubarika Harjana as the reviewer of my dissertation.

This dissertation has gone through several steps, including research experiment, data analysis, writing, and editing. I owe a favour to my lab mates who always cheered up my days in the laboratory. Sokhna, who helped me in days of my adaptation to new environment, my mentor to talk in French and who treated me as her little sister. This also goes to Hugo and Petty for the adorable assistance, Lucie for the warm friendship, and Quan for his kindness. I would also like to say thanks to Haouaria and Laila for their help, Eric for heartfelt welcome. My high appreciation goes to Jean-Michel who always supported me with ideas, advice and lots of journals to read. I am also grateful to other group V members, Dr. Catherine HEMMERT and Prof. Heinz GORNITZKA for the encouragement. Special acknowledgment goes to my colleagues in Universitas Islam Indonesia (UII), particularly to *mas* Anshory who helped me in collecting the plants and extraction process, *mbak* Asih who willingly helped with my administrative inquiry in UPS, and Ms. Heni who has done me a favour to edit and proofread the language in this dissertation.

I would like to say thank you also to the many members of PPI (Persatuan Pelajar Indonesia) Toulouse who always helped and kept me company in my social life there.

My deepest gratitude goes to Kupang research team, *Bu* Eti, *Mbak* Priska and others who made the study possible to begin. I would also like to thank BPOM Kupang for allowing me to use the microbiology laboratory for my research and the Department of Health of Kupang for the research permission.

Last but not least, I do hope this dissertation can be one of the ways to show my respect and gratitude to my honourable parents, in-laws, and siblings. Finally, I dedicate this dissertation to my soul mate as well as friend in sailing over this beautiful, amazing life, my beloved husband, Ruby. May we get a lot of blessings from this struggle.

Whispering my prayers, I wish the aforementioned people and those unstated here, who have helped me in this study and gone through the process with me, receive the blessings and glory from Allah here and after. Amen.

Yogyakarta, July 2017

Arba Pramundita Ramadani

*For indeed, with hardship (will be) ease. Indeed, with hardship (will be) ease.*

*[QS.94 :5-6]*

*A chaque problème il y une solution. S'il n'y a pas de solution, c'est qu'il n'y avant pas de problème.*

*[FBV]*

# TABLE OF CONTENTS

ACKNOWLEDGMENT ..... iii
TABLE OF CONTENTS ..... vi
LIST OF FIGURES ..... viii
LIST OF TABLES ..... ix
LIST OF ANNEXES ..... x
ABSTRACT ..... xi
RESUME ..... xii
INTRODUCTION ..... 1
CHAPTER I ..... 5
LITERATURE REVIEW ..... 5
1. Malaria ..... 5
2. The Life Cycle of *Plasmodium* ..... 6
3. Antimalarial Drug ..... 8
4. Artemisinin-based Combination Therapies (ACTs) in Indonesia ..... 18
5. Antimalarial Drug Resistance ..... 19
6. Medicinal Plants for New Antimalarial Drug Discovery ..... 38
7. Indonesian Medicinal Plants for Malaria Therapy ..... 39
CHAPTER II ..... 51
MATERIALS AND METHODS ..... 51
A. MATERIALS ..... 51
1. Antiplasmodial Activity of Indonesian Medicinal Plants ..... 51
2. Antiplasmodial Activity of Gold (I) Complexes ..... 52
3. K13 Gene and Artemisinin Resistance ..... 53
4. Dihydroartemisinin-Piperaquine Resistance in Kupang ..... 53
B. METHODS ..... 54
1. Antiplasmodial Activity of Indonesian Medicinal Plants ..... 54
2. Antiplasmodial Activity of Gold (I) Complexes ..... 60
3. K13 Gene and Artemisinin Resistance ..... 62
4. Dihydroartemisinin-Piperaquine (DHP) Resistance in Kupang ..... 65
CHAPTER III ..... 70
RESULTS ..... 70
A. Antiplasmodial Activity of Indonesian Medicinal Plants ..... 70

B. Antiplasmodial Activity of Gold(I) Complexes ..... 82
C. Gene Pfk13 and Artemisinin Resistance ..... 94
D. Dihydroartemisinin-Piperaquine Resistance in Kupang ..... 101
CONCLUSION AND PERSPECTIVES ..... 107
A. CONCLUSION ..... 107
B. PERSPECTIVES ..... 108
REFERENCES ..... 110
ANNEXES ..... 119
Annex 1. Plant determination certificate: *T. diversifolia* ..... 120
Annex 2. Plant determination certificate: *C. barbata* ..... 121
Annex 3. Plant determination certificate: *T. crispa* ..... 122
Annex 4. Plant determination certificate: *A. flava* ..... 123
Annex 5. Plant determination certificate: *P. cauliflora* ..... 124
Annex 6. Supplementary materials for publication of K13 ..... 125
Annex 7. Ethic Committee Approval for K13 mutation study in Kupang ..... 154

# LIST OF FIGURES

Figure 1. The life cycle of *P. falciparum* ..... 8
Figure 2. Mode of action of antimalarial drug at the erythrocytic ..... 9
Figure 3. Aminoquinolines: quinine and related compounds ..... 11
Figure 4. Antifolate drugs ..... 12
Figure 5. Artemisinin, a sesquiterpene lactone with a peroxide bridge ..... 14
Figure 6. Miscellaneous compounds ..... 15
Figure 7. The leaves of *T. diversifolia* (a) and tagitinin C structure (b) ..... 41
Figure 8. The leaves of *C. barbata* (a) and cycleapeltine structure (b) ..... 42
Figure 9. The stem of *T. crispa* (a) and one of the aporphine structures, N-formylasimilobine 2-O-β-D-glucopyranoside (b) ..... 43
Figure 10. Stem of *A. flava* (a) and berberine structure (b) ..... 45
Figure 11. The plant of *P. cauliflora* (a) and obaberine structure (b) ..... 46
Figure 12. Structure of [Au(CQ)($PPh_3$)]$PF_6$ ..... 48
Figure 13. Position of Kupang (circled) in the map of Indonesia [208]. ..... 102

# LIST OF TABLES

Table 1. ACTs recommended by WHO ..... 17
Table 2. The dosage of DHP based on body weight classification for *falciparum* malaria ..... 19
Table 3. Resistance genes in antimalarial drug ..... 21

# LIST OF ANNEXES

Annex 1. Plant determination certificate: *T. diversifolia* ..... 120
Annex 2. Plant determination certificate: *C. barbata* ..... 121
Annex 3. Plant determination certificate: *T. crispa* ..... 122
Annex 4. Plant determination certificate: *A. flava* ..... 123
Annex 5. Plant determination certificate: *P. cauliflora* ..... 124
Annex 6. Supplementary materials for publication of K13 ..... 125
Annex 7. Ethic Committee Approval for K13 mutation study in Kupang..... 154

## Various antimalarial strategies in Indonesia to fight *Plasmodium falciparum*


# ABSTRACT

Malaria remains a global public health problem and worsening with the resistance of *Plasmodium falciparum* to Artemisinin-based Combination Therapies (ACTs), the latest and most effective antimalarial drugs. My project aimed to provide insight into malaria elimination in Indonesia. The first part was to look for new antimalarial drugs based on Indonesian ethnobotanical data. Among 25 crude extracts realized on Indonesian traditional medicinal plants, seven showed a good antimalarial activity ($IC_{50}$ < 5µg/mL) and some of them were also active against *Babesia divergens* and *Leishmania infantum*. The second part of the study focused on chemosynthetic organometallic compounds. The structure-activity relationships study on organometallic gold(I)-NHC complexes led to a very active compound on *P. falciparum* with an $IC_{50}$ of 320nM. The third part of this work was dedicated to the study of *P. falciparum* resistance to artemisinin and its derivatives. The correlation between *PfK13* polymorphism and artemisinin resistance has been clearly established thanks to reverse genetic with resistant and sensitive laboratory strains and clinical isolates from Cambodia. This resistance was evidenced *in vitro* throughout a parasite survival assay called $RSA_{(0\text{-}3h)}$. By the same genotypic and phenotypic methods, mapping of *PfK13* polymorphism distribution in Indonesia was performed in Kupang on *P. falciparum* malaria patients. However, at the time of *P. falciparum* blood samples collection, prevalence showed a dramatic decrease hindering the continuation of the clinical study. Facing to the very small number of eligible patients with a *P. falciparum* malaria, no conclusive results has been obtained. In conclusion, medicinal plants and synthetic compounds are potentially interesting as chemical starting point for new antimalarial drugs. Concerning artemisinin resistance, any treatment failure or delayed cure with ACTs has yet to be reported in Indonesia. However, because Indonesia is relatively close to the Southeast Asian areas of resistance, the possible occurrence of such cases in Indonesia must be anticipated by determining the variations of *P. falciparum* malaria chemo-sensitivity and by following *PfK13* polymorphism, responsible for artemisinin resistance.



Keywords : malaria, *Plasmodium falciparum*, traditional medicine, *PfK13*, artemisinin, resistance

# Différentes stratégies en Indonésie pour combattre *Plasmodium falciparum*

## RESUME

Le paludisme demeure un problème de santé publique mondial qui risque de s'aggraver avec la résistance de *Plasmodium falciparum* aux thérapies combinées à base d'artémisinine (ACT), médicaments antipaludiques les plus récents et les plus efficaces. Mon travail avait pour but de proposer différents axes d'élimination du paludisme en Indonésie.

Une première partie a consisté à rechercher de nouveaux médicaments antipaludiques à partir de données ethnobotaniques indonésiennes. Parmi les 25 extraits bruts réalisés à partir de plantes médicinales indonésiennes utilisées traditionnellement dans le traitement du paludisme, sept ont montré une activité antipaludique intéressante ($CI_{50}$ <5 µg/mL) et certains d'entre eux se sont révélés également actifs sur 2 autres pathogènes *Babesia divergens* et *Leishmania infantum*. La deuxième partie de ce travail était axée sur les composés organométalliques synthétiques. Les études de relations structure-activité de ces complexes organométalliques d'or (I) -NHC ont permis de sélectionner un composé actif sur *P. falciparum* avec une $CI_{50}$ de 320nM. La troisième partie du travail a été consacrée à l'étude de la résistance de *P. falciparum* à l'artémisinine et à ses dérivés. La corrélation entre le polymorphisme de *PfK13* et la résistance à l'artémisinine a été clairement établie grâce à des études de génétique inverse avec des souches de laboratoire résistantes et sensibles et des isolats cliniques Cambodgiens. Cette résistance a été mise en évidence *in vitro* par un test de survie parasitaire appelé $RSA_{(0\text{-}3h)}$. Par les mêmes méthodes génétique et phénotypique, la cartographie de la distribution du polymorphisme de *PfK13* en Indonésie a été réalisée dans la zone de Kupang sur des patients infectés par *P. falciparum*. Cependant au moment de la collecte des échantillons de sang, la prévalence de *P. falciparum* a montré une diminution spectaculaire empêchant la poursuite de l'étude clinique. Face au faible nombre de patients admissibles avec un paludisme à *P. falciparum*, aucun résultat concluant n'a pu être obtenu.

En conclusion, les plantes médicinales indonésiennes et les composés synthétiques sont potentiellement intéressants comme point de départ chimique pour de nouveaux médicaments antipaludiques. En ce qui concerne la résistance à l'artémisinine, aucun échec thérapeutique ou parasitologique après traitement par ACT n'a été signalé, pour le moment, en Indonésie. Cependant, les zones de résistance de *P. falciparum* aux ACT dans le Sud-Est asiatique sont relativement proches et nécessitent, en Indonésie, un suivi des variations de la chimiosensibilité du paludisme à *P. falciparum* et du polymorphisme de *PfK13*, responsable de la résistance à l'artémisinine.

Mot de clé : paludisme, *Plasmodium falciparum*, traditionnel médicine, *PfK13*, artemisinin, résistance

# INTRODUCTION

Malaria is a tropical infectious disease, which continues to be one of the largest public health problems in developing countries. According to the World Health Organization (WHO), there were approximately 212 million cases worldwide in 2015 [1]. Most of them occurred in Africa (90%) followed by South East Asia (7%), including Indonesia that shared 9% malaria cases of SEA region in 2015 [1]. In Indonesia, based on the latest data collection, there were 252,027 positive cases reported in 2014 [2]. Although morbidity and mortality statistics are routinely under-reported [3], annual parasite incidence (API) is used to determine the morbidity trend and endemicity area in Indonesia [2]. The latest report from Indonesian Ministry of Health (MoH) stated that the national API in 2015 was 0.85 per 1,000 population. However, several provinces showed higher API level than national level, such as Papua (31.93), West Papua (31.29), East Nusa Tenggara (7.04), Maluku (5.81), North Maluku (2.77), Bengkulu (2.03), and Bangka-Belitung Island (1.08) [4]. Almost 60% of malaria cases in Indonesia are caused by *Plasmodium falciparum* followed by *Plasmodium vivax* (40% approximately) with different proportion in each endemic region [5].

Even though some reports stated minor prevalence in several endemic regions, the malaria remains a constant strike each year [6]. Hence, the continuous effort to eliminate malaria in Indonesia is imperative and many Indonesian scientists explore ethno-botanical data to find efficient medicinal plants allowing the discovery of new antimalarials. However, many studies were promptly stopped often due to un-standardized method of assays or sample preparations. Moreover, several promising medicinal plants are endangered due to lacking of knowledge on its cultivation process and uncontrolled harvesting. All of them make the process of discovering new antimalarials from plants come too far from clinical implementation.

Indeed, exploring medicinal plants remains a common way to discover new drugs. In endemic areas, medicinal plants are the most affordable treatments [7] and compounds such as alkaloids, terpenoids, flavonoids, and others possess antiplasmodial activity [8]. As Indonesia has abundant sources of medicinal plants for traditional disease treatments, people excessively use them until today. For example, malaria is commonly treated using herbal medicinal products, particularly to reduce fever simply by using them as patch, drinking brew preparation or decoction. Several plants naturally growing in Indonesia and widely used to treat malaria include *T. diversifolia, C. barbata, T. crispa, A. flava* and *P. cauliflora*. These plants offer an opportunity to discover new antimalarial drugs through assessment their antiplasmodial activities in conjunction with cytotoxic assays to determine their selectivity. Their activity profile can be extended to some other parasites like *Babesia* close to *Plasmodium* or *Leshmania* for which an overlapping in geographic distribution exists with *Plasmodium* and can be at the origin of co-infections in human [9].

The identification of new chemical entities (NCEs) can be sourced by chemical synthesis [10]. Among synthetic drugs, organometallic compounds, such as gold complexes, are particularly interesting due to the intrinsic activity of the metallic moieties able to increase the efficacy of organic drugs. As a result, it has been published that cationic lipophilic gold (I)-NHC complexes were active against chloroquine-resistant *P. falciparum* strain possibly due to the respective positive influence between cationic gold and the presence of nitrogen or sulfur atoms in the ligands [11].

Apart from discovering new drugs, the Indonesian Ministry of Health (MoH) actively launched the malaria elimination program. Comprehensively, the program covers surveillance and vector control, early diagnosis procedure and fast precise treatments in order to stop malaria spreading [12]. Concerning therapies, the Indonesian government follows and adjusts its treatment policy to the WHO recommendations. Accordingly, Artemisinin-based

Combination Therapies (ACTs) have been implemented as the first line therapy for uncomplicated malaria since 2006 especially as many studies revealed the resistance of *Plasmodium* to non-artemisinin therapies in several regions [13] [14].

ACTs, a combination of artemisinin or one of its derivatives (artesunate, artemether, or dihydroartemisinin) with an antimalarial drug partner belonging to a different chemical class [15], are recommended by the WHO [16, 17]. However, although artemisinin is the most potent antimalarial drug, several studies reported a decreasing parasite susceptibility to artemisinins characterized by delayed parasite clearance times, a sign that artemisinin resistance occur [18-20]. In addition, recent findings figured out that the resistance can be also extended to the companion drug of some ACTs, such dihydroartemisinin-piperaquine (DHP) [21], recently implemented in Indonesia.

The mechanism of artemisinin resistance is different from those involved in other antimalarial drug resistances. This mechanism is based on an entrance in a quiescence state, allowing parasites to survive against high doses of artemisinin at the ring stage. Parasites can resume a regular cycle after the removal of drug [22]. This phenomenon is not observable by a standard chemo-sensitivity assay, but by ring-stage survival assay (RSA), which evaluates the susceptibility of *P. falciparum* to artemisinin [23] and reflects the ability of parasites to survive ART treatment [22]. Further investigations found that mutations of K13 propeller, involved in protein ubiquitination, were correlated to artemisinin resistance [24]. In order to discover new targets to overcome this artemisinin resistance, it is necessary to clarify the role of K13 mutations. This is achieved by both insertion and removal of mutated or wild type *Pfk13* genes on clinical isolates and several *P. falciparum* laboratory lines [25].

Interestingly, for the moment, K13 mutations are found to play a role in artemisinin resistance only in Asia and have been studied and documented in Cambodia, Thailand,

Myanmar, Laos, Vietnam [26, 27], and China [28-32] but are not correlated with any case of resistance in Africa [26, 28]. Since Indonesia lies close to regions where artemisinin resistance emerges and spreads with a strong correlation with K13 mutations, it is highly likely that the phenomenon also occurs in Indonesia. The evaluation of clinical, *in vitro* and genotypic chemo-sensitivity to DHP in Kupang, a region of high malaria prevalence, could provide a new outlook on antimalarial resistance in Indonesia.

# CHAPTER I

# LITERATURE REVIEW

## 1. Malaria

Physicians have diagnosed and treated fevers for thousands of years. Until Robert Koch, Louis Pasteur, and their contemporaries uncovered that “germs” were the cause of most febrile illness, fevers were considered as a disease and not the result of the disease. Malaria-like febrile illnesses (with names like “the ague” or “paludisme”) have been described since Hippocrates as periodic fevers were and associated with marshes and swamps [33]. In-depth malaria studies were feasible after the discovery of the parasite responsible for the illness by Charles Louis Alphonse Laveran in 1880 and the parasite transmission to humans by mosquitoes by Ronald Ross in 1897 [34]. Globally, malaria is a disease caused by a parasite of the genus *Plasmodium*, which is transmitted to human and widespread through female *Anopheles* mosquito bites.

There are five species of human malaria parasite: *Plasmodium falciparum, P. vivax, P. ovale, P. knowlesi* and *P. malariae* which have different world distributions thought overlapping exists. The first symptoms of malaria are nonspecific and similar to influenza (chills, headache, nausea, fatigue, muscle pain, and rigors). The onset of the symptoms is between 1 to 3 weeks after the infection consecutively to a mosquito bite. Fever may appear 2 -3 days after initial symptoms and may follow a pattern of every 2 or 3 days for *P. vivax, P. ovale,* and *P. malariae* infections. With *P. falciparum* fever can be erratic and may not follow specific patterns [35].

*Plasmodium vivax* is mostly found in Middle America, North Africa, and Middle East. The other parasites, *P. ovale* is highly present in Africa and *P. malariae* is widespread around

the world. *P. knowlesi*, a *Plasmodium* species naturally living in macaques, has been positively found in human in Borneo [36], particularly in people living in the natural habitat of simian hosts [37]. *Plasmodium knowlesi* widely exists in west of the Wallace Line in Indonesia (which runs between Borneo and Sulawesi; as well as between Bali and Lombok) [3]. *Plasmodium falciparum*, the deadliest one, is common in West and East Africa, Haiti, the Dominican Republic, part of Amazon (South America), and South East Asia [35] including Indonesia.

Based on the WHO report, malaria caused 429,000 casualties with 303,000 deaths of children under 5 years old in 2015 worldwide [1]. In Indonesia, 80 % malaria cases were found in Papua, West Papua, East Nusa Tenggara (NTT), Maluku, North Maluku and Bengkulu [2, 4]. These provinces are the top-6 provinces with highest malaria rate and have higher level of annual parasite incidence than the national level (0.85/10,000 population) [4]. The Indonesian Ministry of Health stated that in 2014 there were 58 districts /cities considered as high risk areas with 3% population (6,331,381) living there [2]. Furthermore, malaria cases in Indonesia are mainly caused by *Plasmodium falciparum* (60% approximately) and by *P. vivax* (almost 40%) [5].

## 2. The Life Cycle of *Plasmodium*

The life cycle of malaria parasite is complex (Fig. 1). Motile sporozoites are transmitted directly into the human bloodstream by the bite of infected female mosquitoes of the genus *Anopheles* during its blood meal. Within minutes after the bite, the sporozoites circulate briefly in human blood before invading hepatocytes, where for the parasite an asexual life cycle and an important proliferation ensue. Between 5 and 15 days after (depending on the species) thousands of daughter merozoites are released into the blood stream after the rupture of infected

hepatocytes. The merozoites then invade circulating erythrocytes for the clinically important intra-erythrocytic cycle of 48-hour asexual replication [38, 39].

*Plasmodium falciparum* differs from other human malarial parasites species in that infected erythrocytes do not remain in the bloodstream. After a maturation of 24-32 hours parasites passing as ring stage at the trophozoite stage, parasitized erythrocytes adhere to micro-vessel endothelial cells of various organs (called sequestration). Trophozoites then mature into schizonts with an egress of 48 hours after the beginning of the erythrocytic infection, of 16-31 daughter merozoites that invade non-infected erythrocytes to perpetuate the asexual life cycle. Some parasites inside red blood cells differentiate (in response to stress or other cues) into male and female gametocytes. Consecutively to their ingestion by a female mosquito during its blood meal, they undergo a sexual development [38, 39] .

The formation of male and female gametes results in a rapid maturation of the gametocytes in the mosquito gut (10-20 min). One female gamete emerges from one female gametocyte, and three mitotic division cycles give rise to eight male gametes from a single male gametocyte. Fertilization of a female gamete by a male gamete produces a zygote (the only diploid stage), which undergoes rapid meiotic division and mitosis leading to numerous new haploid sporozoites in an oocyst. Then sporozoites migrate to the salivary gland and await introduction into a host during the next blood feeding, thereby ensuring the continuation of the parasite life cycle [40, 41].

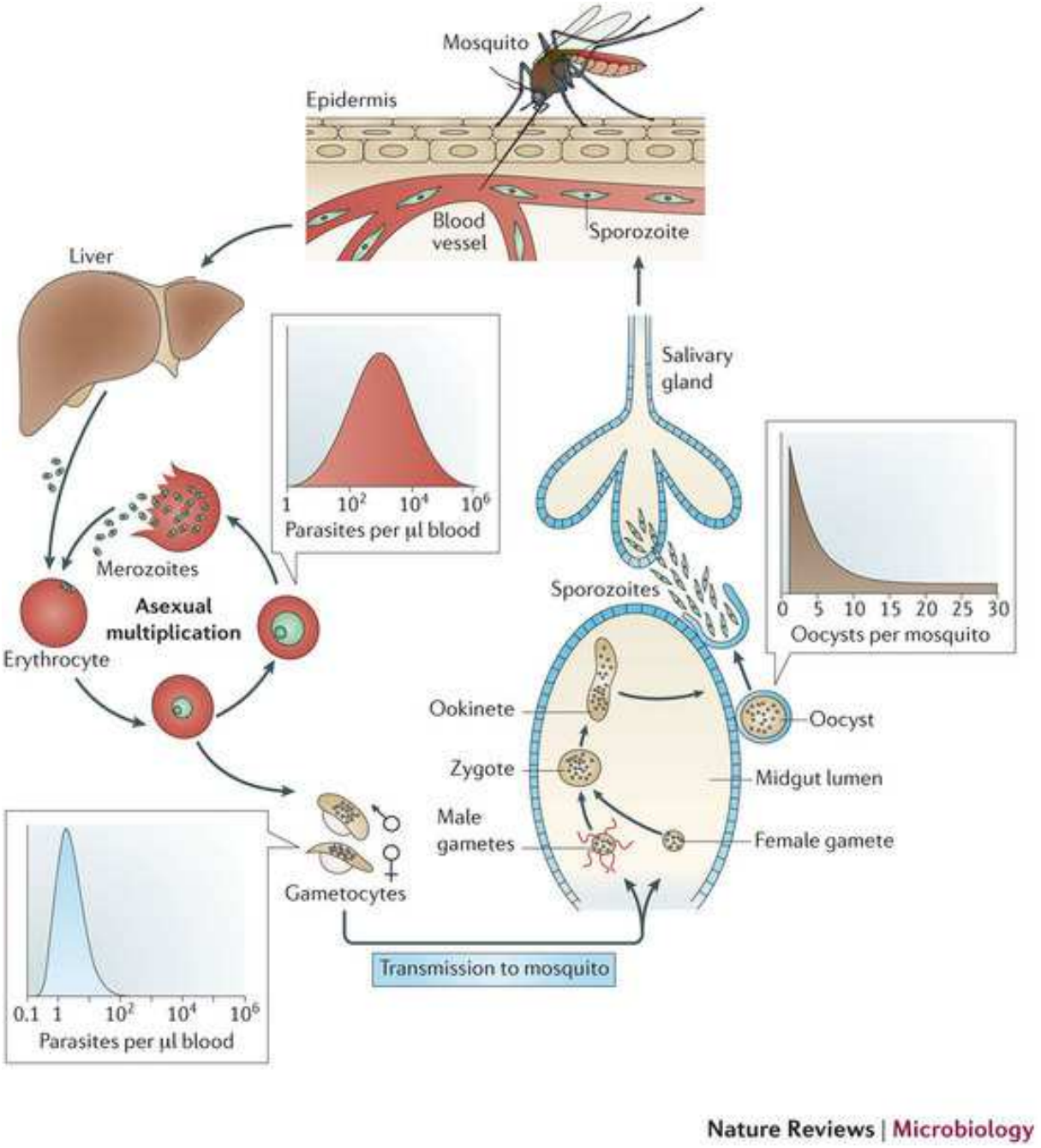



Figure 1. The life cycle of *P. falciparum*
[42]

## 3. Antimalarial Drug

Malaria was among the first diseases to be treated by a pure chemical compound – quinine - isolated from the cinchona bark in 1820. Historically, two herbal treatments for malaria fevers, cinchona bark and qinghao, were used to effectively treat malaria for hundreds

of years. Today, both derivatives of quinine and artemisinin (from qinghao) remain major in the control of malaria [33].

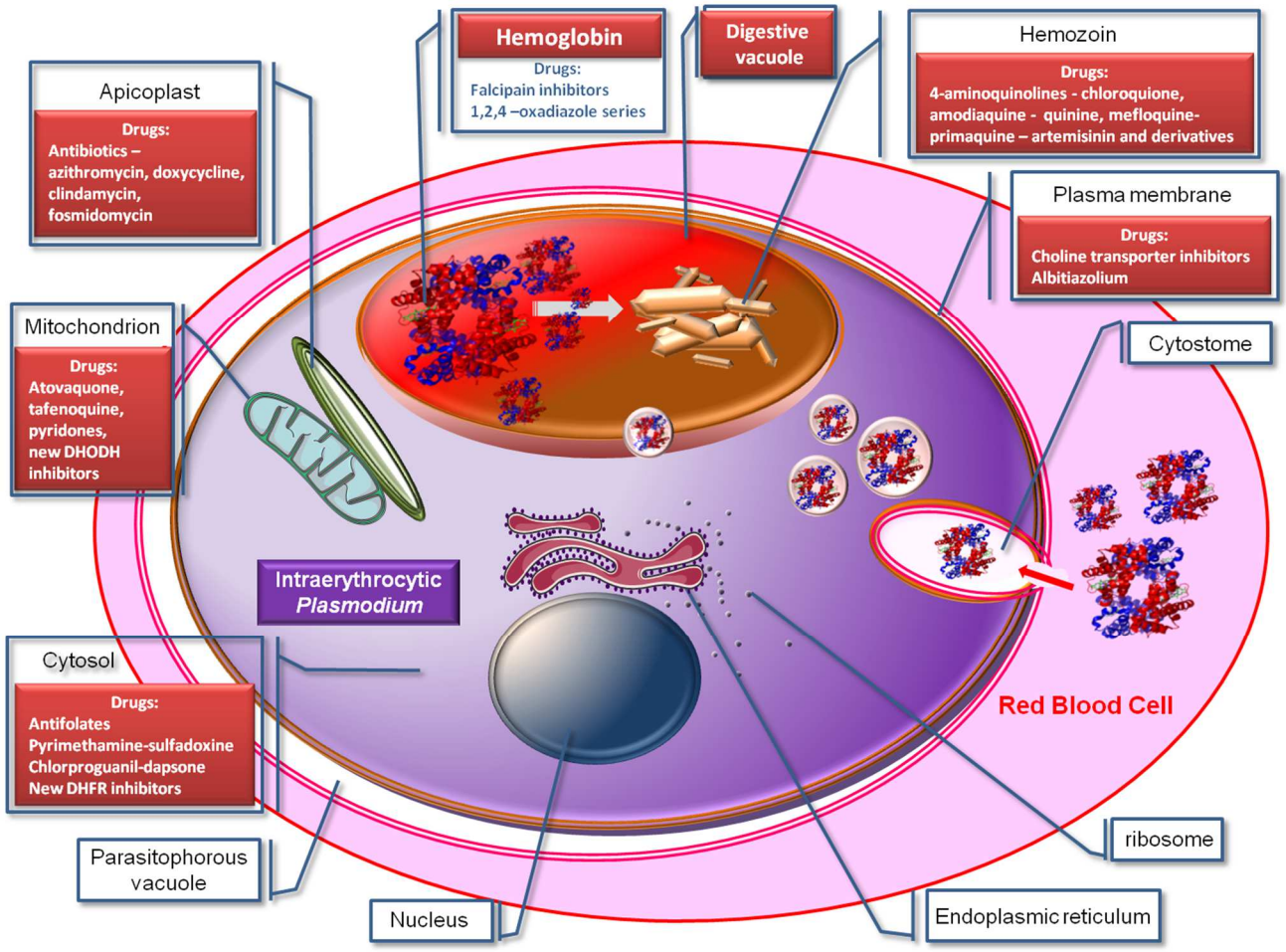



Figure 2. Mode of action of antimalarial drug at the erythrocytic [43-45]

Antimalarials belong to different chemical series with different modes of action (Fig. 2). They target the red blood stage and the merozoite production, some of the gametocytes, and for others also the hepatic stages. In the food vacuole, the polymerization of hemozoin corresponding to the detoxification process of wastes due to haemoglobin digestion is affected by quinolines [43] and artemisinins. Artemisinin and its derivatives are also responsible for alkylation of proteins and heme leading to oxidation damages [46]. At the mitochondrion level, atovaquone, targeting the cytochrome $bc_1$ complex, inhibits the parasitic electron transport chain and respectively the dihydroorotate dehydrogenase (DHODH) linked to the respiratory

chain and implicated in the pyrimidine nucleotides biosynthesis. Antifolates like proguanil and pyrimethamine inhibit the folic acid action involved in the DNA and RNA synthesis by blocking the dihydrofolate reductase and dihydropteroate in the cytosol, (Fig. 2).

a. Quinine and related compounds (Fig. 3)

Quinine, along with its dextroisomer quinidine, continues to be used for the treatment of severe malaria. Chloroquine (CQ) is a 4-aminoquinoline derivative of quinine and the most widely used antimalarial drug. It has been the drug of choice for uncomplicated malaria therapy and for chemoprophylaxis. Yet, its use has dramatically reduced due to widely spread resistance. Chloroquine works by binding with ferriprotoporphyrin IX in the parasite, thereby antagonizing the polymerisation of this toxic metabolic product into inert crystal of hemozoin [47]. Another quinine derivative, amodiaquine, is an available compound closely related to CQ. Other quinine-related compounds in common use include primaquine (specially used to eliminate the exoerythrocytic forms of *P. vivax* and *P. ovale* that cause relapses) and mefloquine, a quinoline-methanol derivative of quinine [48]. The primaquine action mechanism is mediated by reactive metabolites which destroy mitochondrial structure of the parasite [49], while mefloquine is believed to work in the same way as CQ [50].

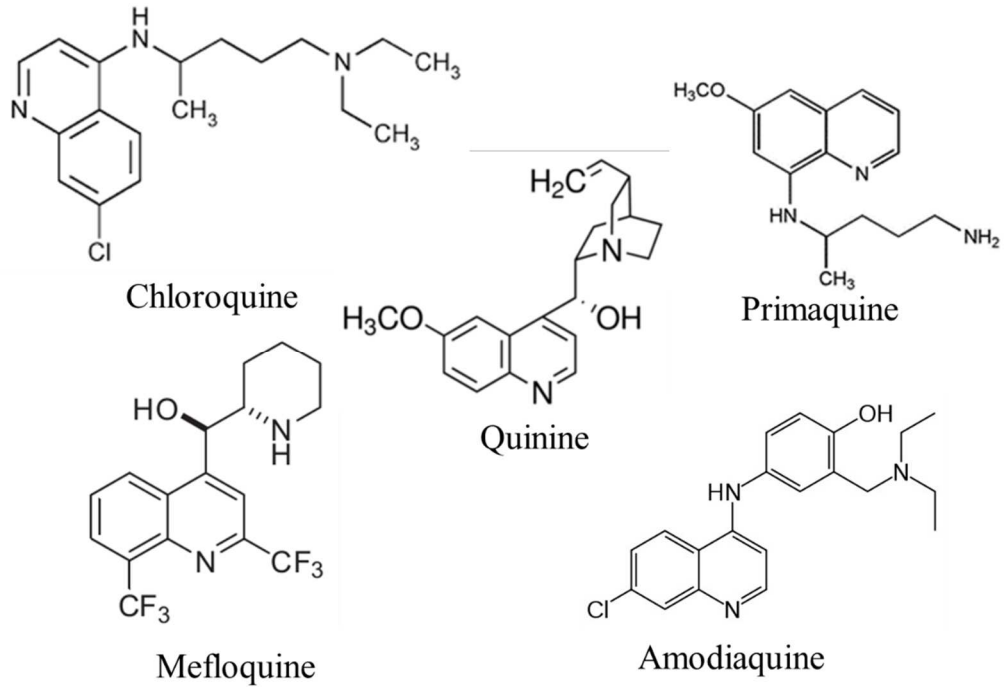



Figure 3. Aminoquinolines: quinine and related compounds

b. Antifolate drugs

These drugs are a variety of dihydrofolate-reductase (DHFR) inhibitors [50] that arrest DNA replication (proguanil, chlorproguanil, pyrimethamine, and trimethoprim) and dihydropteroate synthase (DHPS) inhibitors (dapsone, sulfalene, sulfamethoxazole, sulfadoxine, and others) (fig. 4). Despite their use in combinations, parasite resistance to these drugs can develop rapidly.

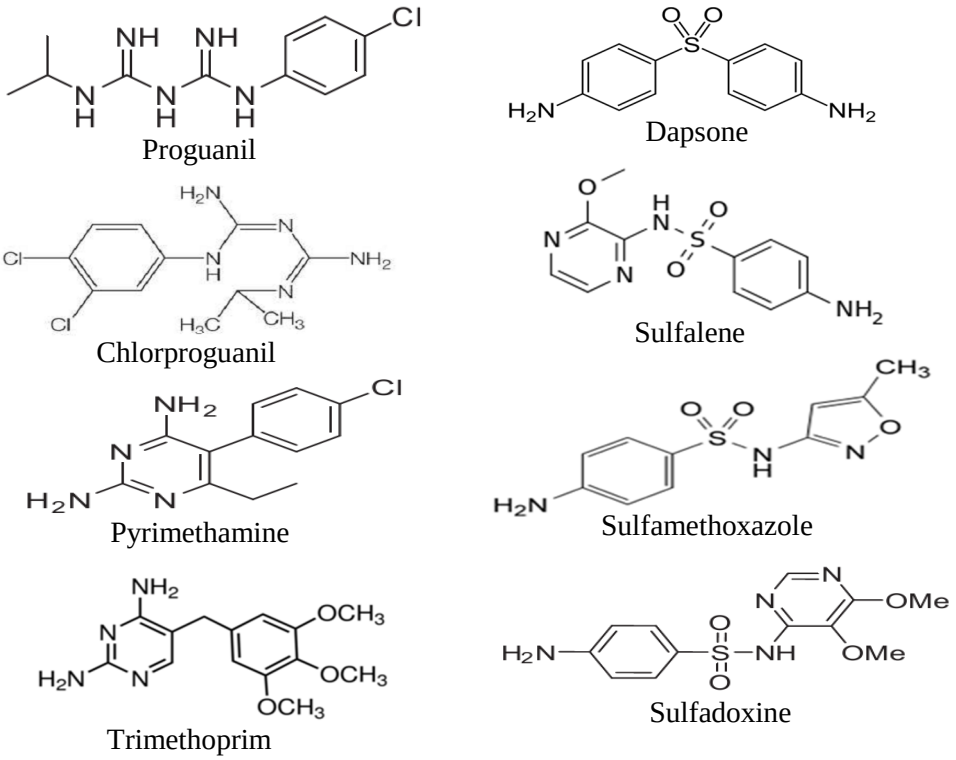



Figure 4. Antifolate drugs

c. Antibiotics

Tetracycline and its derivatives such as doxycycline are very potent antimalarials and used for both treatment and prophylaxis. In areas where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates [51, 52] and effectively reduce the duration of treatment [53]. Another antibiotic, clindamycin, has been used as an effective treatment of malaria when given twice daily for five days but it is a slow-acting drug. With a mean parasite clearance time of four to six days its administration in monotherapy is likely to pose a danger [53]. Therefore, clindamycin treatment in combination with another antimalarial drug is highly recommended. The combination of clindamycin (5 mg/kg Body Weight) with quinine (10 mg/kg Body Weight) is an efficient and attainable choice for the treatment of malaria. Moreover, it shortens the duration of treatment for 3 days twice

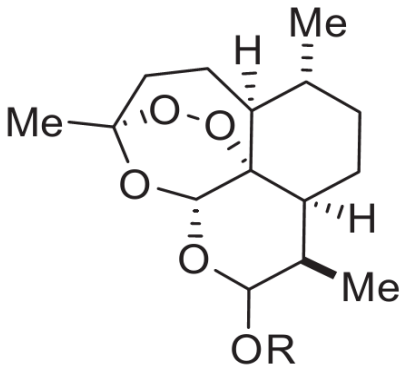
daily and is not contraindicated in children and pregnant women [53, 54]. The combination of clindamycin and CQ is also an effective and plausible treatment even in high rate of CQ resistance regions though requiring a higher dose of CQ [52, 53, 55, 56].

d. Artemisinin

In 1967, the Chinese government initiated the National Project against malaria under the direction project 523 office. Focus on exploration of more than 2000 Chinese herbs has resulted in 640 hits with probable antimalarial activity and 380 extracts studied against animal models of malaria. Promising results were found in *Artemisia annua* L. extracts with strong and reproducible parasite growth inhibitions. These activities were confirmed on animal models (mice and monkeys) and supported the studies which led to the isolation and identification of the active compound named artemisinin (qinghaosu). These remarkable results on *A. annua* activity against malaria parasite were published in 1982 [57].

Pure artemisinin (ART) (Fig. 5) has low solubility in both water and oil but it is possible to administrate it orally, rectally, and intramuscularly [58]. However, for severe malaria patients, oral route is often not possible considering the effect of intense vomiting. In order to overcome this obstacle and to limit the necessity of vegetal supply, several semi-synthetic ART derivatives have been developed [48, 59] like the water-soluble artesunate and the oil-soluble artemether and arteether [60]. The studies of drug metabolism found that ART derivatives are converted into the same active metabolite as artemisinin: dihydroartemisin (DHA) [61, 62]. These compounds are used in monotherapy for treatment of severe malaria and have shown very rapid parasite clearance time and fast fever resolution [48]. Artemisinins probably work by generation

of free radicals when Fe (II) cleaves the peroxide bond of the drug in parasitized red blood cells, followed by alkylation of parasite proteins [49, 63].




Dehydroartemisinin: R = H
Artemether: R = Me
Artesunate: R = -CO-CH$_2$-CH$_2$-CO$_2^-$

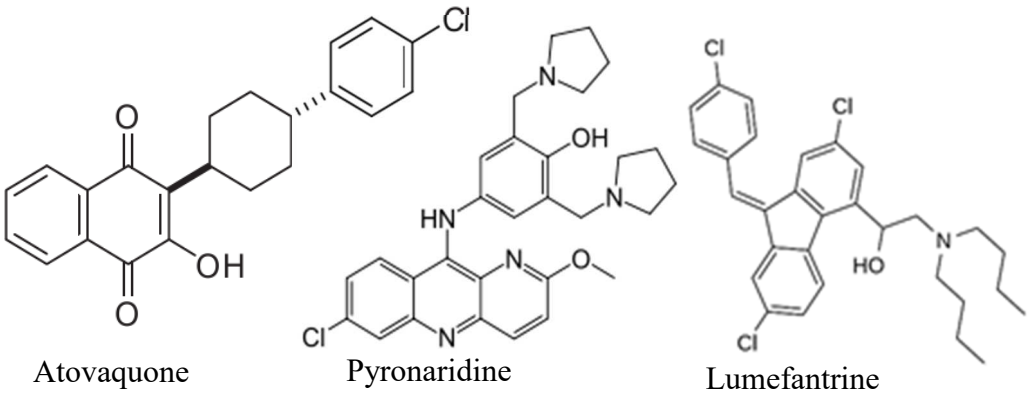
Figure 5. Artemisinin, a sesquiterpene lactone with a peroxide bridge

e. Miscellaneous compounds

Atovaquone, a hydroxynapthoquinone (Fig. 6), is an effective compound against CQ-resistant *P. falciparum*, but when used alone parasite resistance can develop quickly. That is why atovaquone is usually given in combination with proguanil [64, 65]. Atovaquone targets the electron transport chain (ECT) of the mitochondrion, and specifically the cytocrome $bc_1$ complex [49], while proguanil acts not only as DHFR inhibitor but also in a synergistic effect lowering the effective concentration at which atovaquone breaks down the parasite mitochondrial membrane potential [66].

Pyronaridine was reported 100% effective in Cameroon [67] but only 63%-88% in Thailand [68]. Pyronaridine is an acridine-based drug with unknown mode of action, but could act on haemoglobin degradation, haem polymerisation, and topoisomerase-2 activity [50].

Lumefantrine is another antimalarial compound administered in combination with artemether [69, 70]. Lumefantrine is an aryl-amino alcohol [71] that prevents polymerization of haem and free radicals to induce parasite death [72]. In order to prevent recrudescence, it is absorbed and cleared slowly ($t_{1/2}$=3-6 days) [73] to eliminate residual parasite that may remain after artemether has been cleared from the body [72, 74, 75].




Atovaquone Pyronaridine Lumefantrine

Figure 6. Miscellaneous compounds

f. Non-Artemisinin Combination Therapy

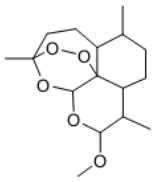
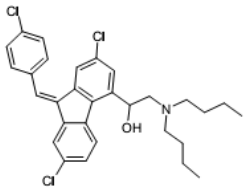
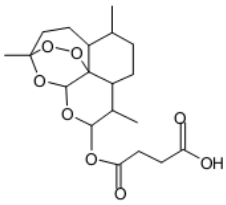
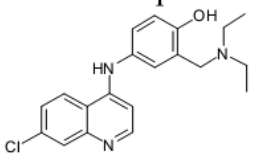
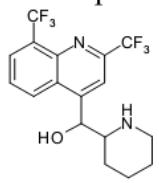
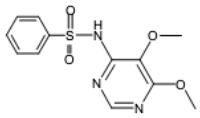
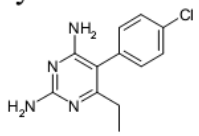
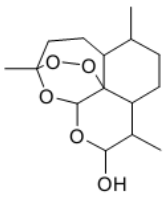
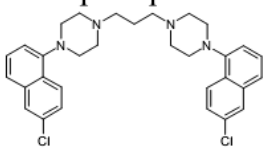
A two-antimalarial combination, especially when they have different mechanisms of action, has the potential to inhibit the development of resistance to each component [48]. The combination of a 4-aminoquinoline drug (CQ or amodiaquine) with sulfadoxine/pyrimethamine (SP) improves the parasitological clearance (compared with SP alone) [76]. However, high levels of resistance against these two chemical series indicate that these combinations are not recommended and no longer used [15].

g. Artemisinin based-Combination Therapies (ACTs)

Another combination therapy approach is artemisinin derivatives associated with longer half-life antimalarials [48]. These combinations have been recommended by the WHO as malaria front-line treatment to counter the resistance of *P. falciparum* to monotherapies and to improve treatment outcome [15].

Artemisinin-based combination therapies (ACTs) are the current standard treatment for uncomplicated malaria. Artemisinin and its derivatives (artesunate, artemether, dihydroartemisinin) are fast acting compounds but are rapidly cleared [15] (human t ½~ 1 hour), and are therefore combined with slow-clearing drugs to kill residual parasites. The typical partner drugs include lumefantrine (human t ½ = 3-4 days) and piperaquine (human t ½ = 8-16 days) [49]. When given in combination with other compounds, a 3-day course of treatment with an artemisinin compound is deemed effective. Five ACTs are currently recommended by the WHO: artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, artesunate + sulfadoxine - pyrimethamine, and dihydroartemisinin - piperaquine [15]. ACTs have supplanted the previously recommended sulfadoxine – pyrimethamine, which in turn replaced CQ treatments [49].

Table 1. ACTs recommended by WHO
[77-80]

| ACTs | Brand name (suppliers) | Artemisinin derivative | Companion drug |
|---|---|---|---|
| Artemether – lumefantrine | Coartem® Riamet® (Novartis) | Artemether  | Lumefantrine  |
| Artesunate – amodiaquine | ASAQ Winthrop® (DNDI/Sanofi) | Artesunate  | Amodiaquine  |
| Artesunate – mefloquine | ASM FDC (DNDI/Cipla Ltd) | | Mefloquine  |
| Artesunate – sulfadoxine – pyrimethamine | Artesunate + Sulfadoxine + Pyrimethamine Tablets (Advacare PHARMA) Malosunate® (Anhui) | | Sulfadoxine  Pyrimethamine  |
| Dihydroartemisinin - piperaquine | Eurartesim® (Sigma-Tau/MMV) Atrketin® (Holleykin) Diphos® (Genix Pharma) | Dihydroartemisinin  | Piperaquine  |

## 4. Artemisinin-based Combination Therapies (ACTs) in Indonesia

The year 2004 was the beginning of ACTs use in the national malaria control program in Indonesia. This was due to the overwhelming burden of resistance of malaria parasites to previous conventional malaria drugs. This step was also in conjunction with WHO's recommendation of using ACTs to fight malaria [81, 82].

The first ACT adopted in Indonesia was artesunate-amodiaquine as first line therapy. This fixed dose regimen is active against *P. falciparum* as well as *P. vivax,* safe for all ages, and affordable though large number of pills to be ingested is a disadvantage. Moreover, in places such as Papua, Lampung, and North Sulawesi or in areas with high chloroquine resistance, treatment failures with artesunate-amodiaquine were reported [82].

Artemether-lumefantrine has been recently, implemented in Indonesia. In a study conducted by Sutanto *et al*. (2013), artemether-lumefantrine proved to be safe and highly efficacious in 59 residents of eastern Sumba Island presenting uncomplicated *P. falciparum* malaria [83]. Another study in southern Papua of Indonesia, an area with multidrug resistant *P. falciparum* also showed high cure rate (95.3%) with artemether-lumefantrine, but it was less responsive to *P. vivax* compared to other combination (43%) [84]. The disadvantages of this ACT are: i) it should be administered twice daily for three days, given with fatty foods, and ii) it is expensive. This limits the utilization of artemether-lumefantrine as second line therapy for *P. falciparum* malaria.

Dihydroartemisinin – piperaquine (DHP) can be another option to overcome the failure of other combinations such as artesunate-amodiaquine [82]. Based on the data of previous ACTs trials in Indonesia, DHP was found to be the best combination to treat uncomplicated malaria in areas with multi-drug resistance. In comparison with all the existing forms of ACTs, a 3-day DHP is the best substitute for the Indonesian ACT program. Moreover, DHP is safe

and effective for both *P. falciparum* and *P. vivax* malaria. The cure rates of DHP reported for the treatment of *P. falciparum* and *P. vivax* were 95.2% and 92.7%, respectively. DHP has been used for more than 2 years in Papua, a high multi-resistance area, as first line therapy [85]. In 2006, the national drug policy was changed and DHP became the first line treatment for any uncomplicated cases of all four parasite species and for treatment of malaria in the second and third trimesters of pregnancy [14].

The dosage of dihydroartemisinin is 2-4 mg/kg of body weight (BW), and 16-32 mg/kg BW for piperaquine. The dose of DHP must be based on body weight rather than on age classification, except if the weight scale is not available.

Table 2. The dosage of DHP based on body weight classification for *falciparum* malaria [86]

| Days | Treatment | Number of tablets per days based on body weight classification | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | ≤ 5 kg | 6 -10 kg | 11-17 kg | 18-30 kg | 31-40 kg | 41-59kg | ≥ 60 kg |
| | | 0-1 month | 2-11 month | 1-4 year | 5-9 year | 10-14 year | ≥ 15 year | ≥ 15 year |
| 1-3 | DHP | ¼ | ½ | 1 | 1 ½ | 2 | 3 | 4 |
| 1 | Primaquine | - | - | ¾ | 1 ½ | 2 | 2 | 3 |

## 5. Antimalarial Drug Resistance

a. Non-Artemisinin Resistance

The obstacle to malaria eradication is parasite resistances to antimalarial drugs. The WHO defines resistance to antimalarials as the ability of parasite strain to survive and/or multiply despite the administration and absorption of a medicine given in doses equal to -or higher than- those usually recommended but within the tolerance of the subject, with the caveat that the form of the drug active against the parasite must be able to gain access to the parasite or the infected red blood cells for the duration of the

time necessary for its normal action [15]. Malaria resistances was reported with chloroquine, antifolates, and atovaquone [87, 88].

Chloroquine resistant *P. falciparum* began to emerge in 1957 in South America, South East Asia to Africa [89]. In the end of 1980, resistances to sulfadoxine-pyrimethamine and mefloquine settled well in Thai-Cambodia and in 2006, resistance to artemisinin appeared [19, 88]. Commonly, the resistance mechanism of antimalarial agents is mediated by multidrug resistance gene polymorphism, for example *Pfmdr1* (chloroquine, mefloquine, amodiaquine, halofantrine, lumefantrine), *Pfcrt* (chloroquine and amodiaquine), *Pfdhps* (sulfadoxine), and *Pfdhfr* (pyrimethamine and proguanil) [90-99].

Two main mechanisms of *Plasmodium* resistance to almost all anti-malarial drugs are: i) modifications in transporter genes reducing drug availability at its site of action or ii) modifications of the drug target by mutations in the corresponding genes (Table 3). For antimalarials targeting the haemoglobin digestion in the food vacuole two transporters are mainly involved in the resistance: the chloroquine resistance transporter (*Pfcrt*) and the multi-drug resistance transporter 1 (*Pfmdr1*). Another possibly related transporter is the multi-drug resistance associated protein (*Pfmrp*) [100]. The mechanism of resistance for these antimalarial agents is mediated by single nucleotide polymorphisms (SNPs) of the genes that encode these transporters or by an increased number of copies of some of them [101, 102]. Modifications of the drug target concern more with antimalarial drugs targeting metabolic pathways, such inhibition of the cellular respiration and the dihydroorotate dehydrogenase (DHODH) involved in the pyrimidine nucleotide biosynthesis at mitochondrion level [103, 104] or the dihydrofolate reductase (DHFR) involved in the production of purines, thymidine and therefore in the DNA biosynthesis in the cytosol [105, 106].

Table 3. Resistance genes in antimalarial drug

| Anti-malarial | | Resistance gene | Main resistance mechanisms | Refs |
|---|---|---|---|---|
| Class | Drug | | | |
| Quinolines | Quinine | *Pfmdr1/other* | Disruption of drug accumulation inside food vacuole by reducing propensity of the drug transporter PfMDR1 to bind to and transfer anti-malarial | [100, 101] |
| | Chloroquine | *Pfcrt/pfmdr1* | Drug extrusion from digestive vacuole by mutating drug transporter PfCRT due to higher lipophilicity and negativity of the transporter allowing ionized chloroquine efflux | [107-109] |
| | Mefloquine | *Pfmdr1/other* | Reduction of parasite susceptibility to mefloquine by amplification of *Pfmdr1* copies | [96] |
| Natural endoperoxide compounds and semi-synthetic derivatives | Artemisinin<br>Artesunate<br>Artemether | *Pfk13* | Quiescence | [22, 24] |
| Antimetabolites | Sulfadoxine - pyrimethamine | *Pfdhps-Pfdhfr* | Modification of the drug target by reducing inhibition of enzymatic activity by the drug | [110] |
| | Proguanil | *Pfdhfr* | | |
| Other | Atovaquone | *Pfcytb* | Modification of the drug target by disruption of cytochrome *bc1* complex | [103] |

b. Artemisinin Resistance

The working definition of artemisinin resistance is based on observations from routine therapeutic efficacy studies of ACTs and clinical trials of artesunate monotherapy. An artemisinin resistance area is suspected by, consecutively to an increased parasite clearance time, ≥ 10% of patient cases with parasites detected 3 days after treatment with ACT and more than 10% of treatment failures on day 28 or 42 [111].

In 2006, declining parasite clearance rates with ART were found, marking an initial detection of ART resistance. This resistance emerged in multiple locations in the Greater Mekong subregion (GMS) and has later triggered another resistance to antimalarial drugs, including partner drugs of ART such as piperaquine [112]. Realizing that ART resistance would be an obstacle for the malaria elimination program, our laboratory team (Group V, LCC-CNRS, Toulouse) reviewed the underlying molecular mechanism of this particular resistance to provide a conceptual framework of cellular network involved in parasite survival. Several cases of ART resistance have been reported. In 2009 *in vitro* studies performed on blood samples of patients with uncomplicated *P. falciparum* malaria suggested an increasing artemisinin failures in western and eastern Thailand, also in Cambodia, apparently correlated with increased artemisinin $IC_{50}$ values (mean of $IC_{50}$ :1.87nM, 3.18nM, and 3.34nM respectively, compare to 1.51nM of artemisinin-sensitive clone W2 [20]. Dondorp's study [19] figured out reduced *in vivo* susceptibility to artesunate in Pailin (western Cambodia) as opposed to Wang Pa (northwestern Thailand). As many as 30% patients followed in Pailin showed 10% recrudescence of artesunate monotherapy, and 5% of them indicated mefloquine combination Wang Pha. This resistance is characterized by slow parasite clearance *in vivo* without corresponding reductions on conventional *in vitro* susceptibility testing, in contrast to the Noedl's observation about increased $IC_{50}$ values the artemisinin in regions with a high failure rate of artesunate-mefloquine.

A number of recent research revealed that the resistance happened to not only artemisinin alone but also with its combinations, such as dihydroartemisinin-piperaquine. In Cambodia parasite recrudescence after dihydroartemisinin-piperaquine treatment was associated with a higher prevalence of *kelch13* mutations

and a higher piperaquine $IC_{50}$. The treatment failure was caused by both artemisinin and piperaquine resistance [21].

It is well established that ART resistance is mediated by mutations of the *Pfk13* propeller which trigger a sequence of events leading parasites to enter in a quiescent state during the presence of ART or its derivatives; the parasite restart its cell cycle after drug elimination. The *Pf*k13 belongs to the kelch super-family of proteins and is likely to have a similar function to Keap 1 protein which plays an essential role in human in protecting against oxidative stress through an unfold protein response (UPR).

A putative transcription factor (uTF) associated with K13 is probably involved in the parasite, such as like the human transcription factor Nrf2 normally linked to keap1. In unstressed conditions Nrf2, linked to keap1, is degraded in the proteasome by a polyubiquitination process [113]. Under stress conditions or in the case of k13 mutations, similar to Nrf2 in human, uTF could be constitutively activated by inhibition of its proteasomal degradation. It was recently found that PfPI3K is a ligand of *Pfk13*. In the case of *Pfk13* mutations, the PI3K ubiquitination is inhibited leading to an increased level of basal PI3P. The PI3P role in ART resistance is confirmed by PI3P transgenic expression that endows artemisinin resistance in non-mutated K13 parasites [114].

The underlying molecular mechanisms linked to K13 mutations and artemisinin resistance have yet to be completely understood. The *in vivo* transcriptome analysis of *Plasmodium falciparum* revealed that artemisinin resistance, caused by single K13 propeller mutations, is associated with increased expression of a network of molecular chaperones and major protein complexes belonging to the Unfold Protein Response

(UPR) pathways. Some of the upregulated genes of these complexes are involved in protein folding and repair in the endoplasmic reticulum (ER) especially BiP (immunoglobulin-binding protein). The misfolded proteins bind to BiP and trigger a cascade of events probably similar to the BIP-PERK-eIF2α pathway observed in mammals and leading to a limitation of global protein synthesis and a cell cycle arrest via cyclin dependent kinases.

The parasite ART-induced quiescence state is characterized by a suspended glycolysis and associated ATP and PEP productions. However, a minimum ATP and PEP production is ensured by apicoplat and mitochondrion that remains active during the quiescence stage [115].

Although the mitochondrial tricarboxylic acid cycle is partly down regulated, the transcription of proteins of the electron transport chain remains active and a FASII pathway is implemented in the apicoplast consecutively after a decrease in host fatty acid incorporations. For those reasons, quiescent parasites can be killed by drugs targeting the respiratory chain of the mitochondrion such as atovaquone, or its FASII metabolism such as haloxyfob, and triclosan.

In conclusion, *Plasmodium falciparum* resistance to artemisinin and its derivatives is not due to a common mechanism of antimalarial resistance but based on the increasing ability of *Pfk13*-mutant parasite to restrain oxidative damages. In a quiescence state, even though main metabolism pathways slow down, the apicoplast and mitochondria keep supplying minimum energy for metabolism to resume growth as soon as the drug is eliminated.

Indeed, the well-defined cellular connections between the different elements involved in artemisinin resistance are a prerequisite to the definition of the best

strategy to fight this resistance and the search for efficient drugs targeting the quiescence phenomenon.

c. A review was published in order to present the whole cellular and molecular network of artemisinin resistance.

L. Paloque, **A. P. Ramadani**, O. Mercereau-Puijalon, J.-M. Augereau, F. Benoit-Vical.

*Plasmodium falciparum*: multifaceted resistance to artemisinins.

*Malaria Journal*, **2016**, 15(1):149. DOI: 10.1186/s12936

Paloque et al. Malar J (2016) 15:149
DOI 10.1186/s12936-016-1206-9

Malaria Journal

REVIEW Open Access

# *Plasmodium falciparum*: multifaceted resistance to artemisinins

Lucie Paloque[1,2], Arba P. Ramadani[1,2,3], Odile Mercereau-Puijalon[4], Jean-Michel Augereau[1,2†] and Françoise Benoit-Vical[1,2*†]

**Abstract**

*Plasmodium falciparum* resistance to artemisinins, the most potent and fastest acting anti-malarials, threatens malaria elimination strategies. Artemisinin resistance is due to mutation of the PfK13 propeller domain and involves an unconventional mechanism based on a quiescence state leading to parasite recrudescence as soon as drug pressure is removed. The enhanced *P. falciparum* quiescence capacity of artemisinin-resistant parasites results from an increased ability to manage oxidative damage and an altered cell cycle gene regulation within a complex network involving the unfolded protein response, the PI3K/PI3P/AKT pathway, the PfPK4/eIF2α cascade and yet unidentified transcription factor(s), with minimal energetic requirements and fatty acid metabolism maintained in the mitochondrion and apicoplast. The detailed study of these mechanisms offers a way forward for identifying future intervention targets to fend off established artemisinin resistance.

**Keywords:** Malaria, Artemisinin-based combination therapy, Resistance, PfK13, Quiescence

## Background

Artemisinin (ART) and its derivatives, introduced in 1980s, are the most potent and fastest acting anti-malarials, producing rapid clearance of parasitaemia and rapid resolution of symptoms. The discovery of artemisinin by Professor Youyou Tu has been rewarded the Nobel Prize for Physiology or Medicine 2015, recognizing that it has radically improved treatment against malaria [1]. *Plasmodium falciparum* artemisinin resistance was first detected in 2006 [2], after decreased clearance rates were observed in patients receiving an artemisinin therapy in clinical studies performed in 2006. It is nowadays widespread across southeast Asia [2, 3]. The delayed clearance of artemisinin-resistant infections exposes larger numbers of parasites to anti-malarial drugs, potentially driving selection to higher-grade artemisinin or to the partner drug. In Asia, treatment failures of artemisinin-based combination therapy (ACT) have recently emerged, including resistance to piperaquine [4]. This represents a serious threat for malaria eradication, widespread artemisinin resistance being predicted to cause in excess of 116,000 deaths annually, with medical costs and productivity losses evaluated as 146 million US$ and 385 million US$ per year, respectively [5]. The resistance to artemisinins is due to mutation of the PfK13 propeller domain endowing the parasites with an increased ability to enter a quiescent state. Piecemeal evidence associates artemisinin resistance with increased unfolded protein response (UPR), dysregulation of the pre-replication phase and the PI3K/PI3P/AKT pathway. Yet, an overall picture about how these modifications result in quiescence-associated artemisinin resistance is lacking. A conceptual framework for the cellular networks involved from oxidative stress to quiescence and parasite survival is proposed here, opening novel avenues for future research.

*Correspondence: francoise.vical@inserm.fr
†Jean-Michel Augereau and Françoise Benoit-Vical contributed equally to this work
[1] CNRS, LCC (Laboratoire de Chimie de Coordination) UPR8241, 205 route de Narbonne, BP 44099, 31077 Toulouse Cedex 4, France
Full list of author information is available at the end of the article

### Rapid overview of antiplasmodial drugs

Currently used anti-malarials belong to different chemical series and all *Plasmodium falciparum* stages can be targeted (asexual red blood cell stages, gametocytes, hepatic stages) by one or another anti-malarial. Each

antiplasmodial drug series has its own specific mode(s) of action. In the food vacuole, haemozoin synthesis, corresponding to detoxification of the waste from haemoglobin by the parasite, is specially affected by quinolines [6] and artemisinins. Artemisinin and its derivatives are also responsible for alkylation of proteins and haem leading to oxidative damages [7]. At the mitochondrial level, atovaquone, targeting the cytochrome $bc_1$ complex, inhibits the parasite electron transport chain and thus the dihydro-orotate dehydrogenase (DHODH) activity linked to the respiratory chain and involved in pyrimidine nucleotide biosynthesis [8]. In the cytosol, inhibition of dihydrofolate reductase (DHFR) by proguanil or pyrimethamine or of dihydropteroate synthase (DHPS) by sulfadoxine [9], blocks the biosynthesis of folate involved in DNA and RNA synthesis. Unfortunately, most anti-malarial drugs have lost their efficacy as resistance has emerged and spread.

The World Health Organization (WHO) defines resistance to anti-malarials as "the ability of *Plasmodium* to survive and/or multiply despite the administration and absorption of a medicine given in doses equal to -or higher than- those usually recommended but within the tolerance of the subject", with the subsequent statement that "the form of the drug active against the parasite must be able to gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" [10]. For some anti-malarial drugs, resistance occurred very quickly after their introduction (Table 1).

Among all mechanisms of resistance described in various organisms, from bacteria, parasites, fungi to human cancer cells, two main mechanisms drive *Plasmodium* resistance to almost all anti-malarial drugs: (i) reduced drug availability at its site of action, essentially due to mutations in transporter genes; and, (ii) modification of the drug target by mutations in corresponding genes (Table 1). Artemisinin resistance results from a different cellular process, quiescence, which is detailed below. The quiescence-based cellular mechanisms comply with the WHO definition of *P. falciparum* drug resistance [10] (see also WHO global report on drug resistance 2010), as artemisinin-resistant parasites survival exposure to therapeutic, lethal concentrations of artemisinin derivatives.

### Recommendations for the use of dual and triple therapy

Parasite resistance to drug monotherapy prompted the WHO to recommend dual or triple therapy, which combines molecules with independent modes of action or distinct target enzymes. Drug combination is usually more effective and in the event of resistance to one component, the second one kills residual resistant parasites. However, some combination therapy, such as sulfadoxine-pyrimethamine plus chloroquine or amodiaquine, must be avoided due to the high levels of resistance established to these drugs already extensively used in monotherapy. For the same reason and because of the rapid acquisition of atovaquone resistance, atovaquone-proguanil is not recommended [2, 11, 12]. Clinical resistance to artemisinin was first detected in Cambodia in 2006 and reported in 2008 [13]. In 2009, Noedl et al. reported artemisinin resistance of uncomplicated falciparum malaria cases in Thailand and Cambodia and correlated delayed clearance with increased in vitro artemisinin $IC_{50}$ values compared to the artemisinin-sensitive clone W2 [14]. Dondorp et al. [15] documented reduced in vivo susceptibility to artesunate in Pailin (western Cambodia) compared to Wang Pha (northwestern Thailand),

**Table 1 Date of introduction and first reports of anti-malarial drug resistance, resistance genes involved [33, 86, 87] and main mechanisms of resistance**

| Anti-malarial drug | Introduced | First reported resistance | Resistance genes | Main resistance mechanisms Refs | |
|---|---|---|---|---|---|
| Quinine | 1632 | 1910 | *Pfmdr1/other* | Disruption of drug accumulation inside the food vacuole by reduced propensity of the drug transporter PfMDR1 to bind to and transfer the anti-malarial | [88, 89] |
| Chloroquine | 1945 | 1957 | *Pfcrt/Pfmdr1* | Drug extrusion on from digestive vacuole by mutated drug transporter PfCRT due to higher lipophilicity and negativity of the transporter allowing ionized chloroquine efflux | [90–92] |
| Proguanil | 1948 | 1949 | *Pfdhfr* | Modification of the drug target: reduced inhibition of enzymatic activity by the drug | [9] |
| Sulfadoxine-pyrimethamine | 1967 | 1967 | *Pfdhps-Pfdhfr* | | |
| Mefloquine | 1977 | 1982 | *Pfmdr1/other* | Reduction of parasite susceptibility to mefloquine by amplification of *Pfmdr1* copy number | [93] |
| Atovaquone | 1996 | 1996 | *Pfcytb* | Modification of the drug target by disruption of Cytochrome *bc1* complex | [8] |
| Artemisinins | 1980s | 2006 | *Pfk13* | Quiescence | [31, 33] |

characterized by a slow parasite clearance time (72–84 h in Pailin and 48–54 h in Wang Pha) and higher failure rates (recrudescence) after artesunate monotherapy (in Pailin 30 % of patients showed recrudescence compared to 10 % in Wang Pha). These altered clinical characteristics were not associated with reduced in vitro susceptibility, contrasting with observations by Noedl et al. [15]. Resistance to artesunate monotherapy first reported in western Cambodia and along the Thailand-Myanmar border, resulted in lengthening of the parasite clearance half-lives from 2.6 h in 2001 to 3.7 h in 2010 [16]. Artemisinin resistance is now also established in Myanmar, Vietnam, Lao PDR, and China [3].

In order to avoid the spread of *P. falciparum* resistance, monotherapy with artemisinin or its derivatives should be banned [17]. ACT has been recommended by the WHO as first-line treatment for uncomplicated malaria since 2001 and is nowadays widely used (Additional file 1). However, in December 2014 eight countries, mainly in Africa (Angola, Cabo Verde, Colombia, Equatorial Guinea, Gambia, Sao Tome and Principe, Somalia, Swaziland) still allowed artemisinin-based monotherapy [18].

Treatment failures with ACT have only been observed in Asia where, in addition to artemisinin resistance, parasites were also resistant to companion drug, e.g. mefloquine along the Thai-Myanmar border [19] or piperaquine in Cambodia [20–22]. In such cases, the ACT used is replaced by another one and the therapeutic efficacy of the replacement ACT must be monitored every 2 years as recommended by the WHO [2] for timely adaptation of the treatment policy. The threshold of 10 % cases with detectable parasites on day 3 after ACT treatment used by the WHO to define areas of artemisinin resistance [2] was established in Asia, a region of low/moderate transmission. In Africa, where transmission is usually much higher, artemisinin resistance has not yet been reported. However, a recent study suggested that a threshold of 5 % cases on day 3 parasite positivity is more suited to artemisinin resistance monitoring in Africa due to higher levels of acquired immunity against *Plasmodium* in African populations contributing to faster parasite clearance [23].

## Phenotypic and genotypic basis of artemisinin resistance

It is striking to note that from chloroquine resistance in 1957 to artemisinin resistance in 2006 [13], all antimalarial drug resistance plaguing Asia was first reported at the Thai-Cambodian border and more especially in Pailin Province. Poor access to medicines, generating traffic of counterfeit drugs, with sub-clinical quantities, use of monotherapy, combined with an intense and extensive migrant labour system, are potential contributors [24–26]. No correlation was found between an increased mutation rate of the parasite genome and drug resistance in these regions, invalidating the hypothesis of the existence of a 'hypermutator' parasite [27]. Resistance in this region has a typical multi-resistance profile, with resistance mutations in *Pfcrt*, *Pfdhps*, *Pfdhfr*, *Pfmdr1* fixed in the parasite populations [28, 29].

Resistance to artemisinins was selected from such multi-resistant parasites circulating in western Cambodian provinces and was shown to be an inheritable genetic trait of the parasites [16, 30]. The mechanism driving artemisinin resistance was discovered using an experimental model, the F32-ART line -a highly artemisinin-resistant line- established in vitro after 5 years of exposure to escalating concentrations of artemisinin [31]. Artemisinin resistance of F32-ART is mediated by a resistance mechanism quite distinct from those previously described [15, 31–33] (Table 1), since these parasites score susceptible using the standard in vitro susceptibility assays [31, 33–35]. *Plasmodium* resistance to artemisinins is due to the enhanced number of young ring forms to enter into a quiescence state upon exposure to artemisinins, and quickly resume growth once the artemisinins are removed. This capacity is conferred by mutations of a gene called *Pfk13* [15, 31, 33]. This gene is now currently monitored to follow artemisinin resistance spread according to WHO recommendations [2, 18, 36].

### *Plasmodium falciparum* resistance to artemisinin is quiescence-based

The first hypothesis of artemisinin resistance based on a partial cytostatic effect of the drug was evoked thanks to a mathematical model in 2000 [37]. The classical definition of quiescence is 'a reversible absence of proliferation,' i.e., a non-dividing cell which eventually restarts its cell cycle when conditions become appropriate. Various quiescent states may depend on the cell history; alternatively, quiescence can be the convergence of an adaptive process to cope with an adverse environment and an active preparation to efficiently resume proliferation [38]. The development cycle of *P. falciparum* presents normal arrest at the sporozoite stage in the mosquito salivary glands or at the gametocyte stage in red blood cells. In vitro, *P. falciparum* cell cycle progression can be strongly delayed at the trophozoite stage in response to isoleucine starvation [39]. A fraction of *P. vivax* and *Plasmodium ovale* sporozoites is able to stop its cell cycle during the hepatic phase to enter into a particular state, named hypnozoites, another form of dormant or quiescent parasites, and responsible for relapses, all under an epigenetic control of gene expression by histone modification enzymes [40].

#### *Quiescence and artemisinin-resistant parasite stages*

Data obtained with the highly artemisinin-resistant F32-ART line and *P. falciparum* isolates showed that artemisinin pressure induces developmental arrest of a sub-population of very young (0–3 h) ring stages which enter a quiescent state, while killing all other stages [31, 41]. Quiescence has also been correlated with K13-mediated artemisinin resistance in field isolates [33].

Quiescent parasites are difficult to identify under the microscope but staining mitochondrial activity with fluorescent dyes, unambiguously labels live quiescent cells in artemisinin-treated cultures [31, 42, 43]. Cell cycle arrest leads to overestimations of the parasite clearance time in patients [44], and a defect of $H^3$-hypoxanthine incorporation in standard chemosensitivity assay leads to an overestimation of the effectiveness ($IC_{50}$) of anti-malarials [31, 42]. As a result, classical susceptibility assays based on parasite proliferation are not suitable to differentiate parasites that are artemisinin-resistant from those that are artemisinin-sensitive. Recrudescence assays and ring stage survival assay ($RSA_{0-3h}$) using highly synchronized *P. falciparum* parasites in culture, based on a 6-h dihydroartemisinin exposure in vitro, followed by culture in drug-free conditions until microscopic read-out at 72 h, are able to detect and quantify artemisinin resistance [31, 35, 41]. Quiescence takes place at a moment of the parasite cycle (young ring stage) where artemisinin is less efficient [43]. Indeed, artemisinin toxicity is potentiated by products of haemoglobin digestion inside the food vacuole, which begins in the mid-ring stage. Haemoglobin degradation products, such as haem and ferrous iron, react with artemisinin to cause oxidative stress and irreversible damages [45]. The low levels of haemoglobin endocytosis and therefore its absence or low digestion level in very young rings, could explain their reduced sensitivity. At this stage, which is approximately 14 h longer in resistant parasites than in wild-type parasites [46], a lower (moderate) oxidative stress is likely more manageable by the parasite, and particularly so by PfK13 mutants, while at the trophozoite stage, artemisinin-dependent damage exceeds the parasite's response abilities [43]. The ring stage arrested development allows parasites to survive during the relatively short period of elevated artemisinin concentration (the elimination half-life in humans varies from one to 11 h depending on the derivative) [31, 41, 47]. Why only a small sub-set population (<1 %) of resistant parasites [42] is able to enter a quiescence stage despite their common genetic pattern remains to be elucidated.

#### *Response of non-mutated PfK13 parasites to artemisinin exposure*

It appears that all parasite lines are able to enter quiescence or dormancy-mediated processes [48]. In vitro, this is monitored by the capacity to proliferate after drug removal, i.e., to recrudesce. Artemisinin-resistance is characterized by a short time to recrudescence as demonstrated with the F32-ART (artemisinin-resistant) and F32-TEM (sensitive) sibling laboratory lines. After exposure to up to 18 µM of artemisinin, F32-TEM was able to resume growth and reach 5 % of parasitaemia 17 days after artemisinin removal compared to 10 days for F32-ART [31]. Other studies reported that several artemisinin-sensitive *P. falciparum* strains restarted their cell cycle from 4 to 25 days after the drug removal [48, 49]. Thus, some wild-type parasites are able to survive artemisinin exposure but artemisinin-resistant strains restart faster with more quiescent parasites and/or quicker awakening [31, 35].

### Involvement of the *Pfk13* locus in *Plasmodium falciparum* resistance to artemisinins

#### *Pfk13 polymorphism*

Whole genome-sequencing of F32-ART and its sibling-sensitive line F32-TEM as well as of artemisinin-resistant *P. falciparum* isolates from Cambodia, allowed artemisinin resistance to be associated with mutations of the K13 protein (accession number PF3D7_1343700), belonging to the kelch super-family of proteins. All mutations found in the *Pfk13* gene and correlated with artemisinin resistance are non-synonymous, and are present after position 440 in the propeller domain (K13-propeller) [33, 36, 50]. Just one of these mutations is sufficient to confer artemisinin-resistance [50]. There is a strong genetic correlation in Asia between K13-propeller mutation and a slow parasite clearance time (half-life longer than 5 h) [3, 28, 33, 36, 51–53]. In Myanmar, among the K13 mutations observed, C580Y and M476I (the mutation acquired by F32-ART parasites) were associated with delayed clearance-time [54]. In Southern China, at the Myanmar border, the single F446I mutation predominates and is associated with delayed clearance [52]. The current picture is that artemisinin-resistant parasites circulating in different geographic areas of Southeast Asia stem from several independent emergences of unique mutations in the K13-propeller domain [28, 36, 53] where the parasite genetic background could play a role in the propensity of K13 mutants to emerge. Three prevalent K13-propeller mutations -C580Y, R539T and Y493H- were shown to correlate strongly with increased parasite clearance in vivo and with increased survival rates in the in vitro $RSA_{0-3h}$ [33, 55, 56]. Gene editing showed that introduction of the wild-type allele into resistant parasites procured a sensitive, very low $RSA_{0-3h}$ survival rate (0.3–0.7 %) whereas the parent isolates harbouring a K13 mutation (R539T, I543T or C580Y) displayed 40–49 % $RSA_{0-3h}$ survival. Conversely C580Y introduced into wild-type

artemisinin-sensitive Cambodian clinical isolates and reference lines conferred varying degrees of in vitro resistance, suggesting additional contribution from the genetic background [50]. Importantly, Asian K13 mutations are generally not found in Africa where numerous additional but rare *Pfk13* alleles were identified [36, 57–59]. An exception is the SNP A578S, observed in many African countries [58–60] as well as Bangladesh [51] and Thailand [36], however this mutation is not associated with artemisinin resistance [36].

Apart from one study in a limited number of African children with severe malaria [61], none of the K13 mutations has been associated with clinical artemisinin resistance for the time being, despite evidence that introducing the C580Y mutation generates artemisinin resistance in vitro in the NF54 parasite strain considered to be of African origin [62]. Genetic determinants of artemisinin resistance have been reviewed recently by Fairhurst [63]. A search of mutations in the *k13 P. vivax* orthologue showed reduced polymorphism compared to *Pfk13* and the V552I mutation identified cannot be currently associated with any *P. vivax* drug resistance [64].

### *PfK13 activity*

The exact function of PfK13 in *Plasmodium* is not known yet, but analogies are possible with the human Keap1 protein function, in particular, in the cell response to oxidative stress (Fig. 1). PfK13 and Keap1 share homologies in the C-terminal BTB/POZ and the 6-kelch propeller domains [33, 65]. The presence of a BTB/POZ domain suggests that PfK13 could dimerize like Keap1. Formation of a dimer has been experimentally confirmed in the recently solved crystal structure of the PfK13 BTB/POZ propeller domain [66]. Based on Keap1 function, a hypothetical model could be that in steady state conditions, the wild-type K13-propeller domain binds to uTF (a putative, unidentified transcription factor functionally equivalent to human Nrf2) allowing its ubiquitination and proteosomal degradation. This ubiquitination could be mediated by one of the *Plasmodium* ubiquitin ligases but this has not yet been investigated. Importantly, the putative uTF transcription factor remains to be identified in the *Plasmodium* genome [67]. Recently, *P. falciparum* phosphatidylinositol 3 kinase (PfPI3K) was shown to undergo a PfK13-dependent ubiquitinylation. It could be immuno-precipitated in a complex with PfK13 [68] and has been included as a complex with PfK13 illustrated in Fig. 1, but direct binding of PfPI3K to PfK13 remains to be demonstrated [68].

### Biochemical consequences of a mutant K13 propeller

Based on Keap1 involvement in human lung cancer [69] and hypertension [70], it is possible to predict that K13-propeller mutations altering the propeller structure impair its biological function and interaction with partner proteins [33]. In particular, K13-propeller mutations would prevent fixation of uTF to the propeller domain, and as a consequence reduce the ubiquitinylation-dependent turnover, promoting translocation of uTF to the nucleus (Fig. 2).

In C580Y mutant parasites, PfPI3K was no longer ubiquitinylated, resulting in 1.5–twofold increased basal PI3P levels. This may reflect disrupted interactions between PfPI3K and PfK13 or disrupted interactions within the PfK13 associated protein complex (Fig. 2). Elevated basal phosphatidylinositol 3 phosphate (PI3P) levels appear central to artemisinin resistance, as K13 wild-type parasites became resistant upon elevation of PI3P levels induced by transgenic expression of human VPS34. Similarly transgenic elevation of PfAKT (also known as protein kinase B) in a wild-type parasite confers in vitro artemisinin resistance in connection with increased levels of PI3P by a feedback mechanism supposed [68]. As PfPI3K enzymatic activity is inhibited by artemisinins, the levels of PI3P likely drop rapidly upon exposure to artemisinins in sensitive parasites, whereas the PI3P level is already elevated in resistant parasites [68]. Additional downstream effectors likely come into play to orchestrate the quiescence response to artemisinin and withstand its toxicity.

PfK13 polymorphisms are also associated with modifications of endoplasmic reticulum homeostasis (Fig. 2). Indeed, transcriptome analysis of *P. falciparum* isolates revealed that artemisinin resistance is associated with increased expression of a network of molecular chaperones and major protein complexes belonging to the UPR pathway. In particular, immunoglobulin-binding protein (BiP) and cyclophilin B (CYPB), belonging to the *Plasmodium* reactive oxidative stress complex involved in protein folding and repair in the endoplasmic reticulum, are upregulated. Along the same line, the endoplasmic reticulum-resident calcium-binding protein (ERC) involved in endoplasmic reticulum $Ca^{2+}$ homeostasis is also overexpressed [71, 72]. This upregulation of UPR in PfK13 mutants likely endows the parasite with increased ability to repair or degrade proteins damaged by alkylation and oxidation generated by artemisinin [73]. Based on homology with the human BiP-PERK-eIF2α pathway involved in the cell cycle arrest under stress conditions (Fig. 3) [74, 75], BiP bound to misfolded/altered proteins could dissociate from the PERK homologue, PfPK4, leading to its activation, and phosphorylation of eIF2α, which could trigger parasite cell cycle arrest via cyclin-dependent kinases, inhibition of protein synthesis, and the translocation of an unidentified transcription factor uTF into the nucleus. In mammalian cells, the PI3K/AKT

**a The human Keap1 complex.** The human Keap1 (Kelch-like ECH associated protein 1) is a repressor protein of Nrf2 (Nuclear factor erythroid 2-related factor 2) activity. The BTB/POZ domain (Broad-Complex, Tramtrack and Bric a Brac or Poxvirus or Zinc-finger domain) and the N-terminal portion of the IVR (Intervening Region) mediate Keap1 homodimerization allowing Cul3 (Cullin 3) binding with BTB domains, and Nrf2 binding, through its ETGE and DLG motifs, with kelch-propeller domains. In basal conditions the Keap1-Nrf2 complex leads to polyubiquitination (Ub) of Nrf2 mediated by the E2-ubiquitin ligase activity of Cul3 promoting Nrf2 degradation by the proteasome. Under stress conditions, modifications of cysteine residues of Keap1 induce a conformational change disrupting the interaction between the ETGE/DLG motifs and Kelch domains, and thus a release of Nrf2, which is no longer degraded and can translocate to the nucleus [96]. Inside the nucleus, Nrf2 controls, through the Antioxidant Response Elements (ARE), the expression of approximately 200 genes involved in the preservation of a healthy intracellular redox balance including the regulation of the expression of molecular chaperones as well as of proteasome subunits. The regulation of Nrf2 activity can also be mediated by the PI3K (phosphatidylinositol 3-kinase) pathway promoting Nrf2 translocation [97]. Thus the Keap1-Nrf2 complex is the major regulator of the cytoprotective response to any endogenous and exogenous stress caused by reactive oxygen species (ROS) and electrophiles. Some mutations of the Keap1-propeller cause a constitutive Nrf2 nuclear translocation increasing chemo-resistance and enhancing tumor cell growth [96]

**b The *P. falciparum* K13 complex.** A putative wild type PfK13 protein complex based on analogy with human Keap1 in steady state conditions. Known features are the capacity of PfK13 to form dimers, presence of PfPI3K in the PfK13 protein complex, and polyubiquitination of PfPI3K in steady state conditions [68]

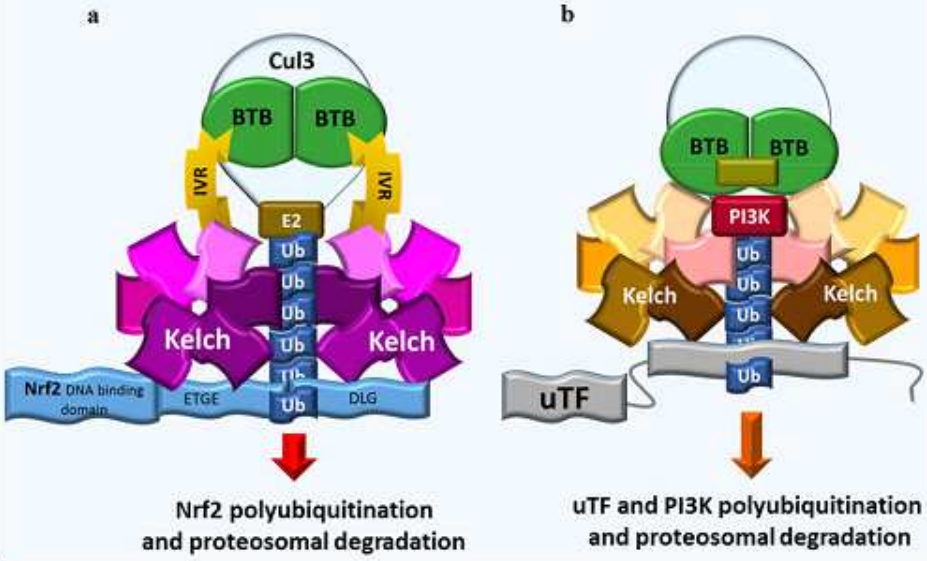



**Fig. 1** The Keap1 complex in human cells and a hypothetical PfK13 complex

pathway participates in UPR regulation and the activated AKT protein (also known as protein kinase B) seems to be required for Protein kinase RNA-like endoplasmic reticulum kinase (PERK)-mediated eIF2α phosphorylation [76]. This is reminiscent of the *Plasmodium* cell cycle slow down and activation of eIF2α kinases (PfeIK1 and PfeIK2) induced by amino acid starvation and observed in sporozoite latency inside mosquito salivary glands [39, 77]. Translocation of uTF could, in addition, be favoured by its phosphorylation by PfPK4 and also, like in humans,

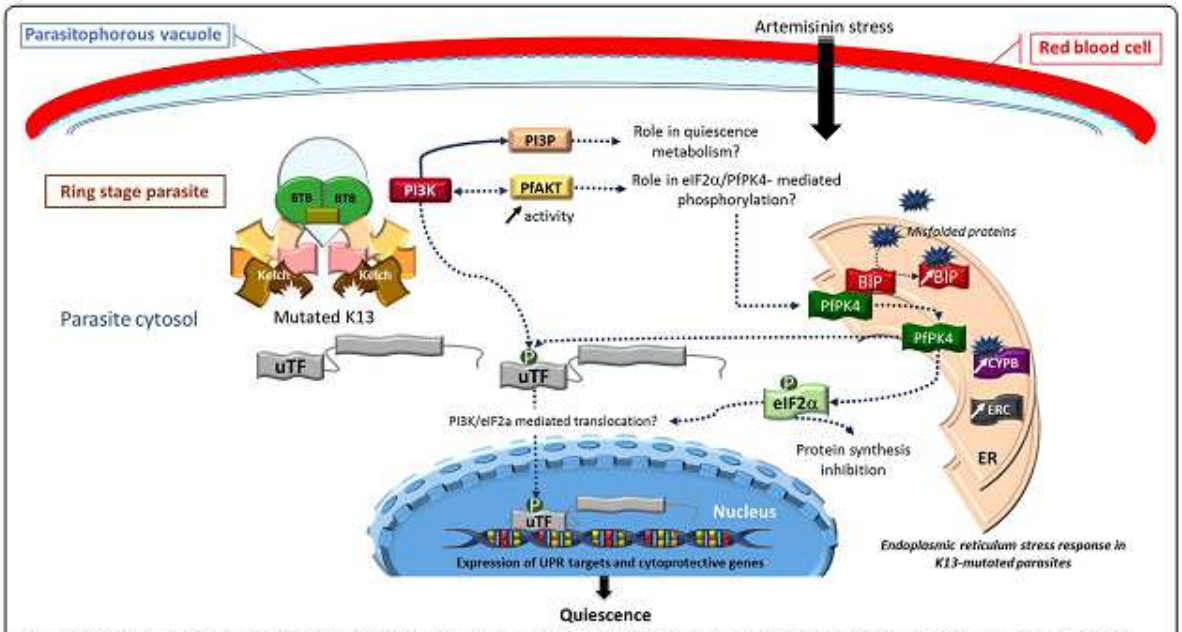



**Fig. 2** Mechanism proposed for PfK13-mediated resistance to artemisinins in K13 mutated *Plasmodium falciparum* at young ring stage. K13-propeller mutation prevents the fixation of uTF and PI3K to the Kelch domain and their ubiquitination, leading to enhanced concentration of PI3P. The artemisinin-induced oxidative stress is responsible for the accumulation of misfolded proteins in the *ER* endoplasmic reticulum. Misfolded proteins bind to BiP, the complex BiP-PfPK4 is dissociated and PfPK4 phosphorylates uTF and eIF2α. eIF2α phosphorylation should allow the translocation of uTF in the nucleus to regulate UPR targets and cytoprotective gene expression and also inhibit protein synthesis [75]. *BiP* immunoglobulin-binding protein, *BTB* broad-complex, tramtrack and Bric a Brac, *CYPB* cyclophilin B, *eIF2α* eukaryotic translation initiation factor 2α, *ERC* endoplasmic reticulum resident calcium binding protein, *PfPK4 P. falciparum* protein kinase 4, *PI3K* phosphatidylinositol 3 kinase, *PI3P* phosphatidylinositol 3 phosphate, *uTF* unidentified transcription factor(s)

by PfPI3K, and could activate the expression of *Plasmodium* UPR and cytoprotective genes (Fig. 2).

This model does not exclude the existence of more than one uTF. Moreover, the PfPK4/PfPI3K downstream regulation cascades remain to be elucidated and experimentally demonstrated (Fig. 2). The involvement of the PfAKT pathway in artemisinin resistance, like PI3P involvement, still remains to be detailed. Moreover, the exact kinetics of events once the parasites are exposed to artemisinin oxidative and alkylating damage needs to be clarified, in particular as PI3K is inhibited by artemisinins [68] and should no longer contribute to refuelling PI3P levels.

Artemisinin resistance possibly includes a combination of the increased degradation of misfolded proteins/repair of damaged proteins especially those involved in the cell cycle progression and the 'PfPK4/PfPI3K' mediated response to the artemisinins' oxidative stress. This cellular response of K13 mutant parasites to artemisinin stress could lead to a sub-set of ring stage parasites to enter into a quiescence state (Fig. 2). Why only a sub-set and a variable fraction of ring stages is involved needs to be understood. It may reflect a finely tuned balance between different cellular effectors that is unequally distributed between individual infected cells.

### Artemisinin-induced quiescence and *Plasmodium* metabolism

Dihydroartemisinin (DHA)-induced quiescence in non-mutated PfK13 parasites is associated with suspended RNA, DNA and protein synthesis [49]. Folate metabolism, isoprenoid metabolism, lactate dehydrogenase activity and glycolysis, the main metabolic pathway producing adenosine triphosphate (ATP) and phosphoenolpyruvate (PEP) in *Plasmodium*, are severely down-regulated [49]. But, even though the metabolism of quiescent parasites is largely down-phased, some pathways remain active and require another source of ATP and PEP, depending on pyruvate production by the apicoplast and on mitochondrial activity (Fig. 4) [49, 78]. In DHA-induced quiescent rings, among the genes that remain actively transcribed, are genes coding for enzymes of the pyruvate metabolism (pyruvate kinase 2, pyruvate dehydrogenase E1 beta subunit), the fatty acid metabolism by FASII -fatty acid synthesis type II- pathway (biotin acetyl-CoA carboxylase, enoyl-acyl carrier

**Bip-PERK-eIF2α pathway**

In mammals the oxidative stress generated in the endoplasmic reticulum (ER) with an accumulation of misfolded proteins causes activation of the Protein kinase RNA-like endoplasmic reticulum kinase (PERK) upon its dissociation from the BiP-PERK complex. PERK directly phosphorylates eIF2α (eukaryotic translation initiation factor 2α) leading to:

i) limitation of the global protein synthesis and degradation of specific proteins like cyclin D1, the regulatory subunit of the cyclin-dependent kinases CDK4 and CDK6 contributing to the induction of G1 cell cycle arrest [98];

ii) translocation of ATF4 (activating transcription factor 4) to the nucleus driving the expression of UPR target and cytoprotective genes [75].

Three eIF2α kinases have been identified in Plasmodium: PfeIK1, PfeIK2 and PfPK4 [99]. PfPK4, the PERK homologue, is known to phosphorylate eIF2α during the different parasite blood stages and plays an important function in the arrest of global protein synthesis in schizont and gametocyte stages [100]. Phosphorylation of eIF2α under ER stress was confirmed in *P. falciparum* after exposure to DTT (dithiothreitol), a disruptor of ER homeostasis leading to the accumulation of unfolded proteins. Although several genes involved in the UPR pathway of other eukaryotes are missing from the parasite, a non-canonical Plasmodium stress response exists, depending on eIF2α phosphorylation by PfPK4 [101]. The Plasmodium CDK-like kinase PfPK5 is activated in the presence of the cyclin Pfcyc-1 and likely participates in the regulation of the nuclear division cycle i.e. at the schizont stage [102].

**Fig. 3** Bip-PERK-eIF2α pathway

reductase/FabI) and for the lipoic acid metabolism (lipoic acid synthase) [49]. The mitochondrial tricarboxylic acid cycle is down-regulated but remains active and genes encoding the proteins of the electron transport chain also remain transcribed at a normal level (cytochrome c subunit II, NADH-ubiquinone oxidoreductase II, flavoprotein sub-unit of succinate dehydrogenase and ubiquinol cytochrome c reductase iron sulfur sub-unit) [49]. This was confirmed by rhodamine staining of parasite after DHA treatment: only rhodamine-positive parasites resumed growth [42]. In stress conditions, depletion of host-derived fatty acids induces an upregulation of the *Plasmodium* FASII pathway [79]. PI3P is present in the apicoplast membrane and can play a role in exchanges of protein and likely lipids [80–82] with the adjacent mitochondrion. The elevated production of PI3P in resistant parasites [68] could play a role in the maintenance of this minimal energetic metabolism based on mitochondrial and apicoplast activity and also found in quiescent resistant parasites [49]. Indeed these parasites cannot survive and resume growth in the presence of atovaquone, an inhibitor of the mitochondrial activity, and the use of haloxyfob, an acetyl-CoA carboxylase inhibitor, as well as triclosan, an inhibitor of FabI delays the recovery of DHA-induced quiescent parasites [35, 42, 49, 83, 84], demonstrating that this minimum active metabolism during the induced quiescence state by artemisinins is essential for the survival and the recovery of the parasites from dormancy.

Thus, the quiescent state induced at the young ring stage by artemisinin in sensitive and resistant parasites likely seems to involve the same cellular mechanisms, but the PfK13 mutation shifts the intra-population distribution so as to allow more parasites to become quiescent in the case of resistant parasites, and to quickly resume growth after the drug removal in vitro or drug elimination in patients.

## Conclusions

It is increasingly clear that the *P. falciparum* resistance to artemisinin and its derivatives is not due to efflux modulation or target modifications as described for other anti-malarials, but is based on increased capacity of PfK13-mutant parasites to manage oxidative damage thanks to greater UPR mobilization. The over-expression of UPR target genes should impact three key points: (an) unidentified transcription factor(s) (uTF) regulating transcription of UPR/oxidative response genes, the PI3 K/PI3P/AKT pathway activity and the PfPK4/eIF2α cascade. This would allow parasite entrance into a quiescence state with minimal energy metabolism provided by the apicoplast and the mitochondrion, maintained by alternative tricarboxylic acid cycle and FASII metabolism, until drug removal/excretion when parasites can resume growth. Recently, the artemisinin-resistant F32-ART line, selected by long-term drug pressure with solely artemisinin, was shown to display an extended age range of stages surviving artemisinin treatment extending to older ring stages (13–16 h) and even trophozoite stages. These parasites were also able to survive lethal doses of diverse classes of anti-malarial drugs, including molecules used as partners in currently recommended ACT, in the

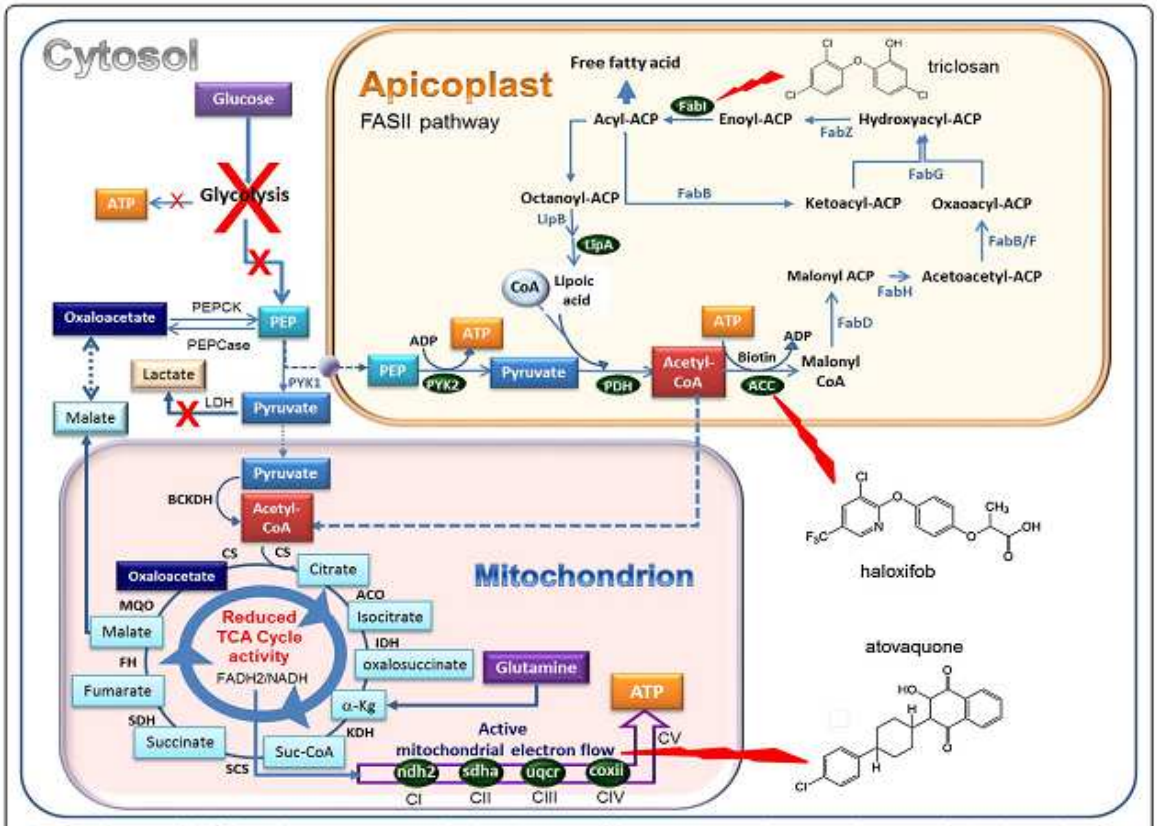



**Fig. 4** Synthetic model of *Plasmodium* quiescence metabolism. Quiescent *P. falciparum* parasites demonstrated an arrested glycolysis pathway leading to suspended production of ATP and phosphoenolpyruvate. Basal metabolism is maintained in quiescent parasites due to FASII metabolism in the apicoplast coupled with ATP production in the mitochondrion. Haloxifob, triclosan and atovaquone can disrupt these biochemical pathways [49, 95]. *Green* protein/enzyme with maintained expression in quiescent rings [49]. *ACC* acetyl-CoA carboxylase, *ACO* aconitase, *BCKDH* branched-chain keto acid dehydratase, *coxii* cytochrome c sub-unit II, *CS* citrate synthase, *FabI* enoyl-ACP reductase, *FabZ* β-hydroxyacyl-ACP dehydratase, *FabG* β-ketoacyl-ACP reductase, *FabB/F* β-ketoacyl-ACP synthase I/II, *FabH* β-ketoacyl-ACP synthase III, *FabD* malonyl-CoA:ACP transacylase, *FASII* fatty acid synthesis type II, *FH* fumarate hydratase, *IDH* isocitrate dehydrogenase, *KDH* α-ketoglutarate dehydrogenase, *LDH* lactate dehydrogenase, *LipA* lipoic acid synthase, *LipB* octanoyl-ACP protein transferase, *MQO* malate quinone oxidoreductase, *ndh2* NADH-ubiquinone oxidoreductase II, *PEP* phosphoenolpyruvate, *PEPCase* PEP carboxylase, *PEPCK* PEP carboxykinase, *PDH* pyruvate dehydrogenase, *PYK* pyruvate kinase, *SCS* succinyl-CoA synthase, *SDH* succinate dehydrogenase, *sdha* flavoprotein subunit of succinate dehydrogenase, *TCA* Tricarboxylic acid, *uqcr* iron sulfur sub-unit of ubiquinol Cytochrome c reductase [49, 83, 95]

absence of the 'classical' mutations of the target genes for these drugs. Thus, long-term in vitro artemisinin exposure selects a novel multidrug tolerant phenotype, which could represent a major threat to anti-malarial drug policy in the field [35]. This threat is exacerbated by the fact that artemisinin-resistant parasites seem able to infect and be transmitted by a large panel of *Anopheles* species, including the major African species *Anopheles gambiae* [85]. This is yet a another reason to urgently clarify the cellular network of ART resistance in order to identify new therapeutic modalities (to protect the molecules currently used), and/or novel drug development, in order to avoid entrance into a quiescent state, target quiescent parasites and promote restart of the cell cycle, rescuing drug susceptibility of parasites.

## Additional file

**Additional file 1.** ACT recommended by WHO and the newly approved combination DHA-piperaquine.

**Authors' contributions**
JMA and FBV designed the review; all authors contributed to acquisition, analysis and interpretation of data. LP, APR, OMP, JMA, and FBV have been

involved in drafting and revising the manuscript critically. All authors read and approved the final manuscript.

**Authors' information**
OM-P and FB-V are co-inventors on the pending patents #US61/904651 and US62/062439, filed by Institut Pasteur. These patents cover the use of K13 mutations as a molecular marker of *P. falciparum* ART resistance.

**Author details**
[1] CNRS, LCC (Laboratoire de Chimie de Coordination) UPR8241, 205 route de Narbonne, BP 44099, 31077 Toulouse Cedex 4, France. [2] Université de Toulouse, UPS, INPT, 31077 Toulouse Cedex 4, France. [3] Present Address: Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. [4] Unité d'Immunologie Moléculaire des Parasites, Institut Pasteur, 75015 Paris, France.

**Acknowledgements**
This study was supported in part by the French Agence Nationale de la Recherche (Grant ANR-13-BSV3-0018-01) and by the French Government programme Investissement d'Avenir, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" (Grant ANR-10-LABX-62-IBEID).

**Competing interests**
The authors declare that they have no competing interests.

Received: 19 December 2015 Accepted: 1 March 2016
Published online: 09 March 2016

**References**

1. Nobel price. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2015/. 2015.
2. WHO. Status report on artemisinin and ACT resistance. Geneva: World Health Organization; 2015.
3. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, et al. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med. 2014;371:411–23.
4. Duru V, Khim N, Leang R, Kim S, Domergue A, Kloeung N, et al. *Plasmodium falciparum* dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations. BMC Med. 2015;13:305.
5. Lubell Y, Dondorp A, Guerin PJ, Drake T, Meek S, Ashley E, et al. Artemisinin resistance–modelling the potential human and economic costs. Malar J. 2014;13:452.
6. Fitch CD. Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs. Life Sci. 2004;74:1957–72.
7. Robert A, Benoit-Vical F, Claparols C, Meunier B. The antimalarial drug artemisinin alkylates heme in infected mice. Proc Natl Acad Sci USA. 2005;102:13676–80.
8. Kessl JJ, Meshnick SR, Trumpower BL. Modeling the molecular basis of atovaquone resistance in parasites and pathogenic fungi. Trends Parasitol. 2007;23:494–501.
9. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev. 2005;57:117–45.
10. Bruce-Chwatt L. Chemotherapy of malaria. Geneva: World Health Organization; 1986. p. 245.
11. Maude RJ, Nguon C, Dondorp AM, White LJ, White NJ. The diminishing returns of atovaquone-proguanil for elimination of *Plasmodium falciparum* malaria: modelling mass drug administration and treatment. Malar J. 2014;13:380.
12. Durand R, Prendki V, Cailhol J, Hubert V, Ralaimazava P, Massias L, et al. *Plasmodium falciparum* malaria and atovaquone-proguanil treatment failure. Emerg Infect Dis. 2008;14:320–2.
13. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med. 2008;359:2619–20.
14. Noedl H, Socheat D, Satimai W. Artemisinin-resistant malaria in Asia. N Engl J Med. 2009;361:540–1.
15. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med. 2009;361:455–67.
16. Phyo AP, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, et al. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet. 2012;379:1960–6.
17. WHO. Guidelines for the treatment of malaria. Geneva: World Health Organization; 2010.
18. WHO. World malaria report 2014. Geneva: World Health Organization; 2014.
19. Na-Bangchang K, Muhamad P, Ruengweerayut R, Chaijaroenkul W, Karbwang J. Identification of resistance of *Plasmodium falciparum* to artesunate-mefloquine combination in an area along the Thai-Myanmar border: integration of clinico-parasitological response, systemic drug exposure, and in vitro parasite sensitivity. Malar J. 2013;12:263.
20. Saunders DL, Vanachayangkul P, Lon C. Dihydroartemisinin-piperaquine failure in Cambodia. N Engl J Med. 2014;371:484–5.
21. Leang R, Taylor WR, Bouth DM, Song L, Tarning J, Char MC, et al. Evidence of *Plasmodium falciparum* malaria multidrug resistance to artemisinin and piperaquine in Western Cambodia: dihydroartemisinin-Piperaquine open-label multicenter clinical assessment. Antimicrob Agents Chemother. 2015;59:4719–26.
22. Spring MD, Lin JT, Manning JE, Vanachayangkul P, Somethy S, Bun R, et al. Dihydroartemisinin-piperaquine failure associated with a triple mutant including kelch13 C580Y in Cambodia: an observational cohort study. Lancet Infect Dis. 2015;15:683–91.
23. WWARN. Clinical determinants of early parasitological response to ACTs in African patients with uncomplicated falciparum malaria: a literature review and meta-analysis of individual patient data. BMC Med. 2015;13:212.
24. Packard RM. The origins of antimalarial-drug resistance. N Engl J Med. 2014;371:397–9.
25. Smith Gueye C, Newby G, Hwang J, Phillips AA, Whittaker M, MacArthur JR, et al. The challenge of artemisinin resistance can only be met by eliminating *Plasmodium falciparum* malaria across the Greater Mekong subregion. Malar J. 2014;13:286.
26. Samarasekera U. Countries race to contain resistance to key antimalarial. Lancet. 2009;374:277–80.
27. Brown TS, Jacob CG, Silva JC, Takala-Harrison S, Djimde A, Dondorp AM, et al. *Plasmodium falciparum* field isolates from areas of repeated emergence of drug resistant malaria show no evidence of hypermutator phenotype. Infect Genet Evol. 2015;30:318–22.
28. Miotto O, Amato R, Ashley EA, MacInnis B, Almagro-Garcia J, Amaratunga C, et al. Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. Nat Genet. 2015;47:226–34.
29. Khim N, Bouchier C, Ekala MT, Incardona S, Lim P, Legrand E, et al. Countrywide survey shows very high prevalence of *Plasmodium falciparum* multilocus resistance genotypes in Cambodia. Antimicrob Agents Chemother. 2005;49:3147–52.
30. Anderson TJ, Nair S, Nkhoma S, Williams JT, Imwong M, Yi P, et al. High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in western Cambodia. J Infect Dis. 2010;201:1326–30.
31. Witkowski B, Lelievre J, Barragan MJ, Laurent V, Su XZ, Berry A, et al. Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. Antimicrob Agents Chemother. 2010;54:1872–7.
32. Imwong M, Dondorp AM, Nosten F, Yi P, Mungthin M, Hanchana S, et al. Exploring the contribution of candidate genes to Artemisinin resistance in *Plasmodium falciparum*. Antimicrob Agents Chemother. 2010;54:2886–92.
33. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature. 2014;505:50–5.
34. Looareesuwan S, Wilairatana P, Viravan C, Vanijanonta S, Pitisuttithum P, Kyle DE. Open randomized trial of oral artemether alone and a sequential combination with mefloquine for acute uncomplicated falciparum malaria. Am J Trop Med Hyg. 1997;56:613–7.
35. Ménard S, Ben Haddou T, Ramadani AP, Ariey F, Iriart X, Beghain J, et al. Induction of multidrug tolerance in *Plasmodium falciparum* by extended artemisinin pressure. Emerg Infect Dis. 2015;21:1733–41.

36. Ménard D, Khim N, Beghain J, Adegnika A, Alam M, Amodu O et al. A worldwide map of *Plasmodium falciparum* artemisinin resistance. N Engl J Med. 2016. (in press).
37. Hoshen MB, Na-Bangchang K, Stein WD, Ginsburg H. Mathematical modelling of the chemotherapy of *Plasmodium falciparum* malaria with artesunate: postulation of 'dormancy', a partial cytostatic effect of the drug, and its implication for treatment regimens. Parasitology. 2000;121:237–46.
38. Daignan-Fornier B, Sagot I. Proliferation/quiescence: the controversial "aller-retour". Cell Div. 2011;6:10.
39. Babbitt SE, Altenhofen L, Cobbold SA, Istvan ES, Fennell C, Doerig C, et al. *Plasmodium falciparum* responds to amino acid starvation by entering into a hibernatory state. Proc Natl Acad Sci USA. 2012;109:E3278–87.
40. Dembele L, Franetich JF, Lorthois A, Gego A, Zeeman AM, Kocken CH, et al. Persistence and activation of malaria hypnozoites in long-term primary hepatocyte cultures. Nat Med. 2014;20:307–12.
41. Witkowski B, Khim N, Chim P, Kim S, Ke S, Kloeung N, et al. Reduced artemisinin susceptibility of *Plasmodium falciparum* ring stages in western Cambodia. Antimicrob Agents Chemother. 2013;57:914–23.
42. Peatey CL, Chavchich M, Chen N, Gresty KJ, Gray KA, Gatton ML, et al. Mitochondrial membrane potential in a small subset of Artemisinin-induced dormant *Plasmodium falciparum* parasites *in vitro*. J Infect Dis. 2015;212:426–34.
43. Klonis N, Crespo-Ortiz MP, Bottova I, Abu-Bakar N, Kenny S, Rosenthal PJ, et al. Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. Proc Natl Acad Sci USA. 2011;108:11405–10.
44. Dogovski C, Xie SC, Burgio G, Bridgford J, Mok S, McCaw JM, et al. Targeting the cell stress response of *Plasmodium falciparum* to overcome artemisinin resistance. PLoS Biol. 2015;13:e1002132.
45. Wang J, Zhang CJ, Chia WN, Loh CC, Li Z, Lee YM, et al. Haem-activated promiscuous targeting of artemisinin in *Plasmodium falciparum*. Nat Commun. 2015;6:10111.
46. Hott A, Casandra D, Sparks KN, Morton LC, Castanares GG, Rutter A, et al. Artemisinin-resistant *Plasmodium falciparum* parasites exhibit altered patterns of development in infected erythrocytes. Antimicrob Agents Chemother. 2015;59:3156–67.
47. Saralamba S, Pan-Ngum W, Maude RJ, Lee SJ, Tarning J, Lindegardh N, et al. Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*. Proc Natl Acad Sci USA. 2011;108:397–402.
48. Teuscher F, Gatton ML, Chen N, Peters J, Kyle DE, Cheng Q. Artemisinin-induced dormancy in *Plasmodium falciparum*: duration, recovery rates, and implications in treatment failure. J Infect Dis. 2010;202:1362–8.
49. Chen N, LaCrue AN, Teuscher F, Waters NC, Gatton ML, Kyle DE, et al. Fatty acid synthesis and pyruvate metabolism pathways remain active in dihydroartemisinin-induced dormant ring stages of *Plasmodium falciparum*. Antimicrob Agents Chemother. 2014;58:4773–81.
50. Straimer J, Gnadig NF, Witkowski B, Amaratunga C, Duru V, Ramadani AP, et al. Drug resistance. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. Science. 2015;347:428–31.
51. Mohon AN, Alam MS, Bayih AG, Folefoc A, Shahinas D, Haque R, et al. Mutations in *Plasmodium falciparum* K13 propeller gene from Bangladesh (2009-2013). Malar J. 2014;13:431.
52. Huang F, Takala-Harrison S, Jacob CG, Liu H, Sun X, Yang H, et al. A single mutation in K13 predominates in Southern China and is associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment. J Infect Dis. 2015;212:1629–35.
53. Takala-Harrison S, Jacob CG, Arze C, Cummings MP, Silva JC, Dondorp AM, et al. Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. J Infect Dis. 2015;211:670–9.
54. Nyunt MH, Hlaing T, Oo HW, Tin-Oo LL, Phway HP, Wang B, et al. Molecular assessment of artemisinin resistance markers, polymorphisms in the k13 propeller, and a multidrug-resistance gene in the eastern and western border areas of Myanmar. Clin Infect Dis. 2015;60:1208–15.
55. Amaratunga C, Witkowski B, Dek D, Try V, Khim N, Miotto O, et al. *Plasmodium falciparum* founder populations in western Cambodia have reduced artemisinin sensitivity in vitro. Antimicrob Agents Chemother. 2014;58:4935–7.
56. Amaratunga C, Witkowski B, Khim N, Menard D, Fairhurst RM. Artemisinin resistance in *Plasmodium falciparum*. Lancet Infect Dis. 2014;14:449–50.
57. Sibley CH. Understanding artemisinin resistance. Science. 2015;347:373–4.
58. Kamau E, Campino S, Amenga-Etego L, Drury E, Ishengoma D, Johnson K, et al. K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. J Infect Dis. 2015;211:1352–5.
59. Taylor SM, Parobek CM, DeConti DK, Kayentao K, Coulibaly SO, Greenwood BM, et al. Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in sub-Saharan Africa: a molecular epidemiologic study. J Infect Dis. 2014;211:680–8.
60. Maiga-Ascofare O, May J. Is the A578S Single-Nucleotide Polymorphism in K13-propeller a marker of emerging resistance to artemisinin among *Plasmodium falciparum* in Africa? J Infect Dis. 2016;213:165–6.
61. Hawkes M, Conroy AL, Opoka RO, Namasopo S, Zhong K, Liles WC, et al. Slow clearance of *Plasmodium falciparum* in severe pediatric Malaria, Uganda, 2011–2013. Emerg Infect Dis. 2015;21:1237–9.
62. Ghorbal M, Gorman M, Macpherson CR, Martins RM, Scherf A, Lopez-Rubio JJ. Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. Nat Biotechnol. 2014;32:819–21.
63. Fairhurst RM. Understanding artemisinin-resistant malaria: what a difference a year makes. Curr Opin Infect Dis. 2015;28:417–25.
64. Popovici J, Kao S, Eal L, Ban S, Kim S, Menard D. Reduced polymorphism in the Kelch propeller domain in *Plasmodium vivax* isolates from Cambodia. Antimicrob Agents Chemother. 2015;59:730–3.
65. Adams J, Kelso R, Cooley L. The kelch repeat superfamily of proteins: propellers of cell function. Trends Cell Biol. 2000;10:17–24.
66. Protein Data Bank. Crystal structure analysis of Kelch protein from *Plasmodium falciparum*. http://www.rcsb.org/pdb/explore/explore.do?structureId=4yy8. 2015.
67. Winzeler EA, Manary MJ. Drug resistance genomics of the antimalarial drug artemisinin. Genome Biol. 2014;15:544.
68. Mbengue A, Bhattacharjee S, Pandharkar T, Liu H, Estiu G, Stahelin RV, et al. A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. Nature. 2015;520:683–7.
69. Padmanabhan B, Tong KI, Ohta T, Nakamura Y, Scharlock M, Ohtsuji M, et al. Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. Mol Cell. 2006;21:689–700.
70. Boyden LM, Choi M, Choate KA, Nelson-Williams CJ, Farhi A, Toka HR, et al. Mutations in kelch-like 3 and cullin 3 cause hypertension and electrolyte abnormalities. Nature. 2012;482:98–102.
71. Morita M, Sanai H, Hiramoto A, Sato A, Haraoka O, Sakura T, et al. *Plasmodium falciparum* endoplasmic reticulum-resident calcium binding protein is a possible target of synthetic antimalarial endoperoxides, N-89 and N-251. J Proteome Res. 2012;11:5704–11.
72. Mok S, Ashley EA, Ferreira PE, Zhu L, Lin Z, Yeo T, et al. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. Science. 2015;347:431–5.
73. Liu Y, Lok CN, Ko BC, Shum TY, Wong MK, Che CM. Subcellular localization of a fluorescent artemisinin derivative to endoplasmic reticulum. Org Lett. 2010;12:1420–3.
74. Gosline SJ, Nasamento M, McCall LI, Zilberstein D, Thomas DY, Matlashewski G, et al. Intracellular eukaryotic parasites have a distinct unfolded protein response. PLoS One. 2011;6:e19118.
75. Kansanen E, Jyrkkänen H-K, Levonen A-L. Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. Free Radical Biol Med. 2012;52:973–82.
76. Blaustein M, Perez-Munizaga D, Sanchez MA, Urrutia C, Grande A, Risso G, et al. Modulation of the Akt pathway reveals a novel link with PERK/eIF2alpha, which is relevant during hypoxia. PLoS One. 2013;8:e69668.
77. Zhang M, Fennell C, Ranford-Cartwright L, Sakthivel R, Gueirard P, Meister S, et al. The *Plasmodium* eukaryotic initiation factor-2alpha kinase IK2 controls the latency of sporozoites in the mosquito salivary glands. J Exp Med. 2010;207:1465–74.
78. Salcedo-Sora JE, Caamano-Gutierrez E, Ward SA, Biagini GA. The proliferating cell hypothesis: a metabolic framework for *Plasmodium* growth and development. Trends Parasitol. 2014;30:170–5.
79. Botte CY, Yamaryo-Botte Y, Rupasinghe TW, Mullin KA, MacRae JI, Spurck TP, et al. Atypical lipid composition in the purified relict

plastid (apicoplast) of malaria parasites. Proc Natl Acad Sci USA. 2013;110:7506–11.
80. Bhattacharjee S, Stahelin RV, Speicher KD, Speicher DW, Haldar K. Endoplasmic reticulum PI(3)P lipid binding targets malaria proteins to the host cell. Cell. 2012;148:201–12.
81. Tawk L, Chicanne G, Dubremetz JF, Richard V, Payrastre B, Vial HJ, et al. Phosphatidylinositol 3-phosphate, an essential lipid in *Plasmodium*, localizes to the food vacuole membrane and the apicoplast. Eukaryot Cell. 2010;9:1519–30.
82. Kobayashi T, Sato S, Takamiya S, Komaki-Yasuda K, Yano K, Hirata A, et al. Mitochondria and apicoplast of *Plasmodium falciparum*: behaviour on subcellular fractionation and the implication. Mitochondrion. 2007;7:125–32.
83. Shears MJ, Botte CY, McFadden GI. Fatty acid metabolism in the *Plasmodium* apicoplast: drugs, doubts and knockouts. Mol Biochem Parasitol. 2015;199:34–50.
84. Ralph SA, van Dooren GG, Waller RF, Crawford MJ, Fraunholz MJ, Foth BJ, et al. Tropical infectious diseases: metabolic maps and functions of the *Plasmodium falciparum* apicoplast. Nat Rev Microbiol. 2004;2:203–16.
85. St Laurent B, Miller B, Burton TA, Amaratunga C, Men S, Sovannaroth S, et al. Artemisinin-resistant *Plasmodium falciparum* clinical isolates can infect diverse mosquito vectors of Southeast Asia and Africa. Nat Commun. 2015;6:8614.
86. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. Epidemiology of drug-resistant malaria. Lancet Infect Dis. 2002;2:209–18.
87. Witkowski B, Berry A, Benoit-Vical F. Resistance to antimalarial compounds: methods and applications. Drug Resist Updat. 2009;12:42–50.
88. Sanchez CP, Dave A, Stein WD, Lanzer M. Transporters as mediators of drug resistance in *Plasmodium falciparum*. Int J Parasitol. 2010;40:1109–18.
89. Koenderink JB, Kavishe RA, Rijpma SR, Russel FG. The ABCs of multidrug resistance in malaria. Trends Parasitol. 2010;26:440–6.
90. Ibraheem ZO, Abd Majid R, Noor SM, Sedik HM, Basir R. Role of different Pfcrt and Pfmdr-1 mutations in conferring resistance to antimalaria drugs in *Plasmodium falciparum*. Malar Res Treat. 2014;2014:950424.
91. Summers RL, Nash MN, Martin RE. Know your enemy: understanding the role of PfCRT in drug resistance could lead to new antimalarial tactics. Cell Mol Life Sci. 2012;69:1967–95.
92. Setthaudom C, Tan-ariya P, Sitthichot N, Khositnithikul R, Suwandittakul N, Leelayoova S, et al. Role of *Plasmodium falciparum* chloroquine resistance transporter and multidrug resistance 1 genes on in vitro chloroquine resistance in isolates of Plasmodium falciparum from Thailand. Am J Trop Med Hyg. 2011;85:606–11.
93. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number. Lancet. 2004;364:438–47.
94. Ubben D, Poll EM. MMV in partnership: the Eurartesim(R) experience. Malar J. 2013;12:211.
95. Ke H, Lewis IA, Morrisey JM, McLean KJ, Ganesan SM, Painter HJ, et al. Genetic investigation of tricarboxylic acid metabolism during the *Plasmodium falciparum* life cycle. Cell Rep. 2015;11:164–74.
96. Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL. The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. Redox Biol. 2013;1:45–9.
97. Nakaso K, Yano H, Fukuhara Y, Takeshima T, Wada-Isoe K, Nakashima K. PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. FEBS Lett. 2003;546:181–4.
98. Raven JF, Koromilas AE. PERK and PKR: old kinases learn new tricks. Cell Cycle. 2008;7:1146–50.
99. Fennell C, Babbitt S, Russo I, Wilkes J, Ranford-Cartwright L, Goldberg DE, et al. PfeIK1, a eukaryotic initiation factor 2alpha kinase of the human malaria parasite *Plasmodium falciparum*, regulates stress-response to amino-acid starvation. Malar J. 2009;8:99.
100. Zhang M, Mishra S, Sakthivel R, Rojas M, Ranjan R, Sullivan WJ Jr, et al. PK4, a eukaryotic initiation factor 2alpha(eIF2alpha) kinase, is essential for the development of the erythrocytic cycle of *Plasmodium*. Proc Natl Acad Sci USA. 2012;109:3956–61.
101. Chaubey S, Grover M, Tatu U. Endoplasmic reticulum stress triggers gametocytogenesis in the malaria parasite. J Biol Chem. 2014;289:16662–74.
102. Doerig C, Endicott J, Chakrabarti D. Cyclin-dependent kinase homologues of *Plasmodium falciparum*. Int J Parasitol. 2002;32:1575–85.

## 6. Medicinal Plants for New Antimalarial Drug Discovery

Malaria control is based on different strategies, among others are developing an effective vaccine, eradicating mosquito-vectors and discovering new drugs [116, 117]. In fact, the development of vaccine proved to be very problematic. Moreover, the attempt to control *Anopheles* mosquito populations has had a limited success, although the use of insecticide bed nets is highly correlated to malaria death rates reduction [118]. The limitation of vaccine and vector control as well as the increasing resistance of malaria parasites to antimalarial drugs pointed the importance of the discovery of new antimalarial agents [119]. The main concern over antimalarial drug development, beyond the antimalarial activity, is that the drugs must be affordable for the population in endemic regions of developing countries [7, 120].

Regarding these problems, plant-derived compounds play an important role in new antimalarial drug discovery. The knowledge of medicinal plants by native people of malarious regions can initiate medicinal chemistry exploration based on specific antimalarial clinical data. Many plants were screened or are under evaluation for their antiplasmodial activities [121-123], in the image of the discovery of quinine from *Chincona* bark [124] and artemisinin from *Artemisia annua.*

When the Spanish and Portuguese began to colonize South America, they discovered the use of the bark of *Chinchona* tree by native Indians to treat fevers such as those caused by malaria. Tea made from the bark cured the people suffering from malaria. The main antimalarial compound was isolated from the bark of *Cinchona succiruba* (Rubiaceae) in 1820 and identified as quinine, one of the 31 alkaloids contained in the plant [125].

The search for new antimalarial agents continued and found qinghaosu or artemisinin, a sesquiterpene isolated from *Artemisia annua* (Asteraceae), a plant used in Chinese traditional

medicine to treat fevers including those provoked by malaria [126, 127]. Artemisinin has proven to be effective in chloroquine-resistant strains of human malaria [128].

Another species used as an antimalarial drug in Chinese traditional medicine is *Dichroea febrifuga* (Saxifragaceae) [129] with the active principle, febrifugine. It has been evaluated clinically against *P. vivax* and *P. ovale* but its liver toxicity has limited its use for malaria treatment [130]. The NAPRALERT natural product database has listed plant species from 152 genera from popular usages for antimalarial properties in Africa, Americas, and Asia [131].

Antiplasmodial activities of plant crude extracts can be classified based on the $IC_{50}$ criteria proposed by Basco and collaborators. Good activity refers to an $IC_{50} < 10$ µg/mL, $10 < IC_{50} < 50$ µg/mL is classified as moderate, $50 < IC_{50} < 100$ µg/mL indicates low activity, and $IC_{50} > 100$ µg/mL is considered inactive [132, 133]. For others, inactive criteria are reached if the $IC_{50} > 100$ µM, $20 < IC_{50} < 60$ µM is classified as low activity, and $1 < IC_{50} < 20$ µM refers to moderate or limited activity [134]. A recent study gave another classification but with closed criteria. Very good activity refers to an $IC_{50} < 2$ µg/mL, $2.0 < IC_{50} < 5.0$ µg/mL is classified as good, $5.1 < IC_{50} < 10$ µg/mL indicates good to moderate, $11 < IC_{50} < 25$ µg/mL is determined as weak, $26 < IC_{50} < 50$ µg/mL is specified as very weak, and $IC_{50} > 100$ µg/mL is considered inactive [135].

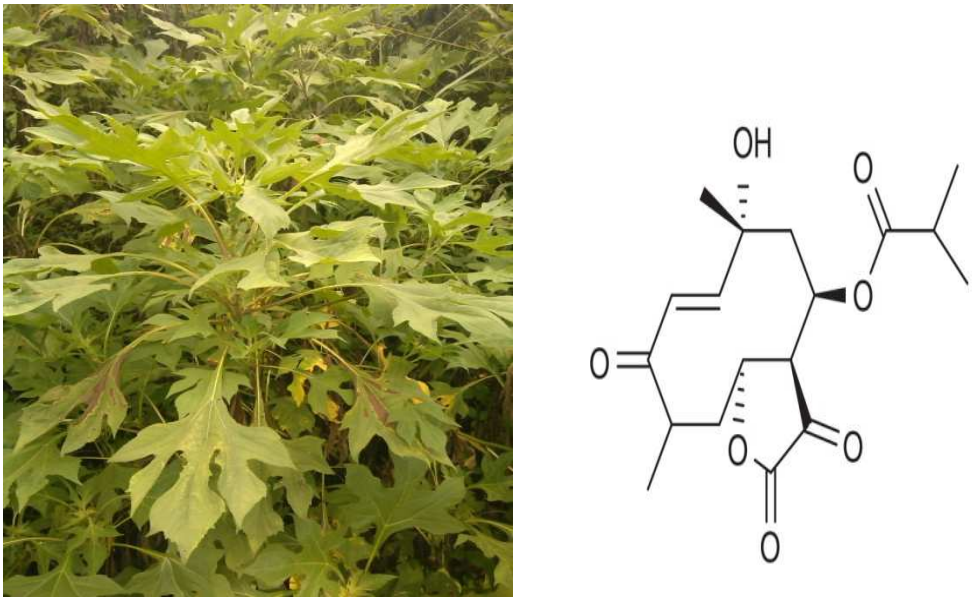
## 7. Indonesian Medicinal Plants for Malaria Therapy

Indonesia is rich in medicinal plants widely used by the population for any diseases including malaria. Basically, the use of medicinal plants for malaria treatment is based on fever therapy like in South America with *Cinchona* or *Artemisia* in China. Some studies hypothesized that antimalarial plants may act on unique biochemical targets for protozoa [131].

The ethno-botanical approach is the most common strategy for screening plant activities. From plants, belonging to various families, which are traditionally used for malaria fever therapy and naturally grow in Indonesia, five species were selected as potential sources of antimalarial agents due to their widely well-known use and for some of them are not well chemically characterized: kembang bulan (*Tithonia diversifolia* (Hemsl.) A. Gray), cincau rambat (*Cyclea barbata* Miers), brotowali (*Tinospora crispa* (L.) Hook. F. & Thomson), kayu kuning (*Arcangelisia flava* Merr), and sengkubak (*Pycnarrhena cauliflora* (Miers.) Diels).

a. *Kembang bulan* (*Tithonia diversifolia* (Hemsl.) A. Gray)

*Tithonia diversifolia* is a woody herb or succulent shrub locally called *kembang bulan* and also known as Mexican sunflower. It is characterized by opposite leaves, attenuate base, acute apex, and crenate margin. It belongs to the kingdom Plantae (plants), subkingdom Tracheobionta (vascular plants), superdivision Spermatophyta (seed plants), division Magnoliophyta (flowering plants), class Magnoliopsida (dicotyledons), subclass Asteridae, order Asterales, family Asteraceae, genus *Tithonia*, and species *Tithonia diversifolia* (Hemsl) A. Gray [136]. It is commonly used for several traditional medication purposes including malaria fever [137]. Its infusion is suitable for constipation, stomachache, indigestion, sore throat, and liver pain [138]. Some research showed that its ethanolic leaf extract exhibited anti-malarial activity [139]. In addition, the ether extract from its aerial parts demonstrated an *in vitro* antiplasmodial activity ($IC_{50}$: 0.75μg/mL on chloroquine-sensitive strain (FCA)). This finding led to extraction of active compound, the sesquiterpene lactone tagitinin C with 0.33μg/mL $IC_{50}$ against *Plasmodium* FCA strain [140]. Moreover, *in vivo* study showed that the ethanol extract of *T. diversifolia* is active *in vivo* at 200 mg/kg BW in a malaria Swiss albino mice model [141].




**a** **b**

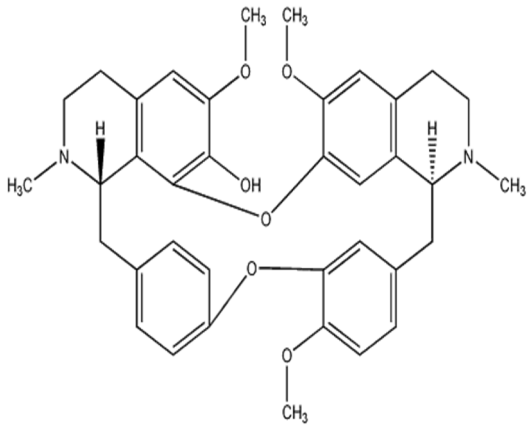
Figure 7. The leaves of *T. diversifolia* (a) and tagitinin C structure (b)

b. *Cincau rambat* (*Cyclea barbata* Miers)

*Cyclea barbata* (Menispermaceae) can be easily distinguished by its deltoidovate, hispid leaves with acuminate apex, finely mucronate acumen, and long male and female inflorescences with dense capitate flowers. It is in the kingdom Plantae, division Magnoliophyta, class Magnoliopsida, order Ranunculales, family Menispermaceae, genus *Cyclea*, and species *Cyclea barbata* Miers [136]. In Java, the leaves of *C. barbata* are typically prepared in the form of jelly consumed as stomach medicine and a brew prepared from the dried roots is used as a prophylactic against fever [142]. It has been shown that *C. barbata* leaves had a protective effect on aspirin-induced gastric ulcer in Balb/c mice [143]. The alkaloid extract of *C. barbata* roots demonstrated both antimalarial and cytotoxic activities, and among the five active bisbenzylisoquinolines alkaloids that were isolated, the most active was cycleapeltine with $ED_{50}$ of 29 and 41 ng/mL on *Plasmodium* strains D-6 and W-2 respectively [144]. Continuous studies of the alkaloidal fraction of *C. barbata* roots led to identification of two new bisbenzylisoquinoline alkaloids: (-)-2'-

norlimacine and (+)-cycleabarbatine [145] but their antimalarial properties were not evaluated.

a




b

Figure 8. The leaves of *C. barbata* (a) and cycleapeltine structure (b).

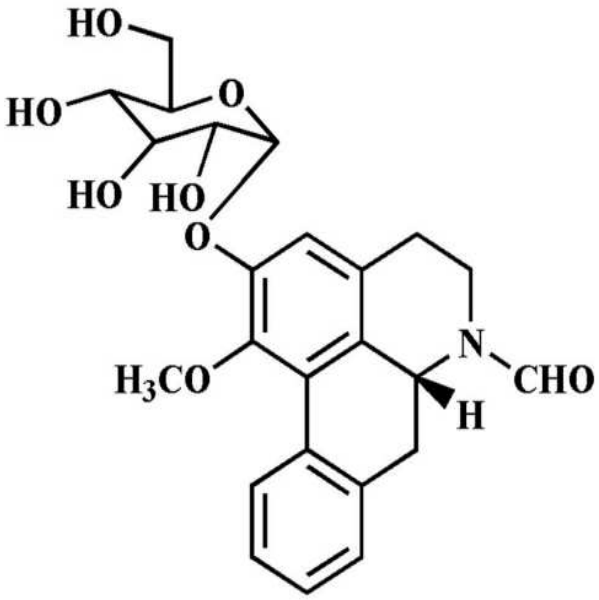
c. *Brotowali* (*Tinospora crispa* (L.) Hook. F. & Thomson)

*Tinospora crispa* (L.) (Menispermaceae) is a tropical liana (woody) with heart shaped large shiny green leaves and yellow coloured flowers which are fascicled [146]. This plant is predominant in primary rainforests or mixed deciduous forests of South East Asia and Africa including Thailand, Malaysia, and Indonesia [147]. It is endemic in several areas of Indonesia particularly in Java. This plant is part of the kingdom Plantae, phylum Tracheophyta, class Magnoliopsida, order Ranunculales, family Menispermaceae, genus *Tinospora*, and species *Tinospora crispa* (L) Hook. F. & Thomson [148]. *T. crispa,* known as brotowali in traditional folklore, is used for various therapeutic purposes such as treatment for diabetes, hypertension, stimulation of appetite and protection from mosquito bites, fever and malaria, stomachache and jaundice. An infusion of the stems is

prescribed as a worming, and a decoction of the whole plant is used as a general tonic [147, 149-151].

Phytochemical investigations of the stems of brotowali led to the isolation of aporphine alkaloid, including N-formylasimilobine, N-formylanonaine, N-formyldehydroanonaine, N-formylnomuciferine, magnoflorine, paprazine, N-trans-feruloyltyramine, and cytidine [152, 153]. Further investigation revealed that methanol extract of *T. crispa* displays an antioxidant activity [154-156], methanol crude extract of *T. crispa* shows antiproliferative activity [156, 157], aqueous extract possesses hypoglycemic effect [149], and ethanol extract and its isolated fraction stimulate murine macrophage cell (RAW264.7) viability and intracellular expressions of cytokines [158], and stem extract of *T. crispa* shows both *in vitro* antiplasmodial activity [159] and *in vivo* against *P. berghei* in dose-dependent manner [160].

a




b

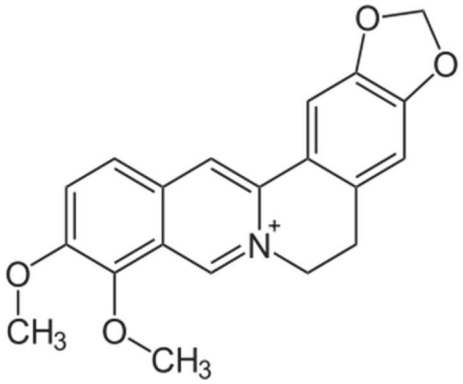
Figure 9. The stem of *T. crispa* (a) and one of the aporphine structures, N-formylasimilobine 2-O-β-D-glucopyranoside (b)

d. *Akar kuning* (*Arcangelisia flava* Merr)

*Akar kuning* (*A. flava)* found in Sumatra, Java, Borneo, and Sulawesi in Indonesia is a large, woody, glabrous, dioecious liana up to 20m long. The stem is up to 5 cm in diameter, yellow wood, and exuding yellow sap when cut. It is a member of the kingdom Plantae, division Magnoliophyta, class Magnoliopsida, order Ranunculales, family Menispermaceae, genus *Arcangelisia*, and species *Arcangelisia flava* Merr [161]. Traditionally, the sap of cut stems of *A. flava* is drunk to treat typhoid fever and sprue [162], hepatitis, stomach disturbance, and malaria [163]. *A. flava*, also known as katola by the communities of Muna district in Southeast Sulawesi, is traditionally used as antidiarrheal and yellow fever treatment by Wawoni tribes.

Some alkaloids yielded from *A. flava* are berberine, 8-hydroxyberberine, columbamine, jatrorrhizine, palmatine, thalifendine, dehydrocorydalmine, shobakunine, (-)R,R-limacine, (+)R,S-homoaromaline, and pycnarrhine [162]. A previous study mentioned that berberine of *A. flava* has antimicrobial activity [164], antioxidant and cytotoxic activity [165]. Its antiplasmodial activity is also figured out against *P. falciparum* with $IC_{50}$ ranging from 0.4 to 8.6 µg/mL [166]. In addition, the berberine extracted from *A. flava* inhibits telomerase activity in *P. falciparum* as a potential target for future malaria chemotherapy [167].

a




b

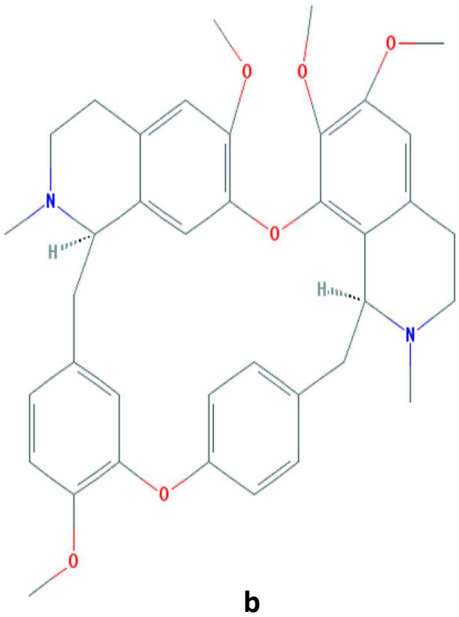
Figure 10. Stem of *A. flava* (a) and berberine structure (b)

e. *Sengkubak* (*Pycnarrhena cauliflora* (Miers.) Diels)

Sengkubak (*Pycnarrhena cauliflora* (Miers.) Diels) from West Borneo has been used widely by Dayak tribes as a cooking flavor and treatment for flatulence (by placing soaked leaves on the stomach), fever and malaria (by drinking leaf infusion). This plant can be found in primary or secondary forests with spatial distribution and tend to grow in clusters. It is a slender twining shrub and frequently climbing up on a tall tree [168]. Sengkubak belongs to the kingdom Plantae, phylum Magnoliophyta, class Magnoliopsida, order Ranunculales, family Menispermaceae, genus *Pycnarrhena*, and species *Pycnarrhena cauliflora* (Miers.) [148]. The bisbenzylisoquinoline alkaloid compounds of *P. cauliflora*, like obaberine, picnarine [169], are responsible for many activities including antioxidant and cytotoxic properties [170, 171] but the antimalarial activity has yet to be

experimentally proved. From the eight species in the genus *Pycnarrhena,* only four (*P. australiana, P. ozantha, P. manilensis, P. longifolia*) have been deeply investigated.

a




b

Figure 11. The plant of *P. cauliflora* (a) and obaberine structure (b)

All these five selected plants are naturally growing in Indonesia. *T. diversifolia, C. barbata,* and *T. crispa* are easily found in gardens or in cultivation centers. *A. flava* and *P. cauliflora* are found in forests. These species are now endangered despite the clinical interest of some of them, such as *A. flava* which has been studied for its numerous activities and *P. cauliflora* that is considered as a promising source of medicinal plants. Deforestation for various reasons, and limited direct economic benefits for the people are the main factors of their extinction. In order to confirm the antiplasmodial activity and to increase their economic benefit, those selected plants were collected and extracted, and their activities were investigated using standard *in vitro* assays with various parasites and human cell lines for cytotoxic studies. Further study on isolation and characterization of active compounds will be required before developing a new antimalarial drug.

## 8. Gold Complexes as Antimalarial Agents

Metal-based therapeutic agents have become an increasingly important research effort toward the development of new compounds with less toxic side effects and with an understanding of their mechanism of action [172-176]. In particular, the use of metal containing drugs as antiparasitic agents has not been very much explored. The emergence of resistance to antiparasitic drugs induces an urgent need for new, effective, non-toxic anti-parasitic drugs. Moreover, an immense number of rational combinations of appropriate organic molecules (used in traditional medicine) with different transitional metal-drug synergism can enhance the activity of parental organic drugs due to bonding with the metal ion. The stabilization of the drug by coordination with the metal ion may be related to this activity enhancement that leads to a prolong contact of the drug with the organism and more efficient biological targeting. Furthermore, the toxicity diminution of the metal ion may occur since its complexation with the organic drugs makes it less available for toxic reactions [177].

The same approach, metal-drug synergism, has also been applied for antimalarial metal agent discovery. It was reported that in 1987, using the drugs primaquine and amodiaquine, 32 metal complexes were synthesized with two of those being based on gold (III). The activity study proved that all compounds were active against *P. falciparum* as well as the parental drugs [178].

Gold is among the most ancient of all metals used in medicine and its current use has allowed information regarding toxicological and clinical administration to become available along with valuable studies concerning its metabolism and molecular targets. An attempt of attaching gold-containing fragment to CQ showed a strong variation of the electronic properties. Moreover, the diversity of possible gold-ligand fragments that can be attached to base CQ or chloroquine diphosphate (CQDP) yields a widespread exploration of their anti-malarial properties [178].

One of the CQ-metal complexes, $[Au(PPh_3)(CQ)]PF_6$ potentially inhibited the growth of *P. berghei* and was also very effective against two CQ-resistant FcB1 and FcB2 strains of *P. falciparum*. It has been documented that the activity was 5-10-fold grater than the corresponding CQDP, displaying that gold coordination leads to a significant enhancement of the activity of the parental drug against resistant parasites without any adverse reaction or apparent acute toxic responses observed [179]. The principal mechanism proposed for the antimalarial action of $[Au(CQ)(PPh_3)]PF_6$ against resistant strains of *P. falciparum* is the interaction with heme and the inhibition of β-hematin formation. Both of the enhanced activity and the ability of this compound to lower CQ-resistance are related to the high lipophilicity of the metal complex and the important structural modification of the CQ structure imposed by the presence of the metal-containing fragment [180].

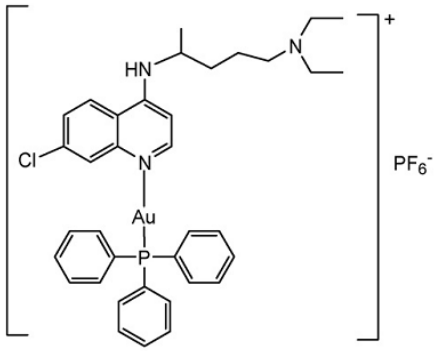



Figure 12. Structure of $[Au(CQ)(PPh_3)]PF_6$

This result then stimulates a derivatization process of CQ-Au complex by structure modification of $[Au(CQ)(PPh_3)]PF_6$ complex. The modification included variation of the phosphine ligand with the purpose of inducing changes in the electronic and steric properties, variation of counter anion, variation of the gold oxidation state (both Au(I) and Au(III)), and use of other biologically important ligands. The result demonstrates that Gold (III) complexes are more active than CQDP against CQ-resistant strain K1. The highest activity for this series

is found for complex [Au(Pet3)(CQ)]PF6, which is 5-fold more active than CQDP against CQ-resistant strain FcB1 [178].

Recently, another ligand widely studied for its coordination with gold complex is NHC (NHC = *N*-heterocyclic carbene). NHCs represent one of the most important classes of ligands in organometallic chemistry. The easy preparation of NHC-precursors has allowed an almost infinite access to new organometallic complexes, in which the nitrogen atoms of the azoliums rings can be functionalized by organic groups, organometallic moieties and biological entities [11]. Moreover, because of their easy preparation via Ag carbene transfer route, the interest in $Au^{I}$-and $Au^{III}$-NHC complexes has surged in the past decade [181] and interesting biological activities of numerous NHC-metal complexes have been documented in several articles [182].

$Au^{I}$-and $Au^{III}$-NHC compounds are generally readily prepared, stable to air and moisture, and some of them, especially dinuclear gold(I) and polynuclear gold(I)-heterometal species display long-lived intense photoluminescence at room temperature [181]. In particular, given that these ligands are extremely good σ-donors, they form strong Au-carbene bonds, giving stable $Au^{I}$-NHC complexes that are insensitive to biologically important thiol groups. $Au^{I}$-NHC have shown potential medical applications [183], especially as anticancer [182, 184-187], antiarthritis [188], and antimicrobial agents [189]. It has to be mentioned that gold-based compounds, including some gold(I)-NHCs, show anti-mitochondrial activity with great specificity [183]. In addition, pharmaco-modulations of *N*-functionalized bis(imidazolum) proligands and their corresponding silver(I), gold(I) and gold(III) complexes leading to the second series of complexes with $IC_{50}$ values up to 330nM against *P. falciparum* strain, without any hemolysis [190].

The study by Micale and colleagues explored the possible mechanism of gold complexes as antimalarial agents. It was noted that the parasite enzyme Falcipain 2 (Fp2) may

involve in this regard. This protein is correlate to the degradation of host's hemoglobin that produce significant amounts of peptide and amino acids for parasite growth. This process is strongly occurring at the early throphozoite stage. Although the direct correlation of gold compounds to Fp2 inhibition has been investigated *in vitro,* further findings displayed that the inhibition of Fp2 only represent one of the diverse mode of action of gold compounds inhibit *P. falciparum* replication [11, 191].

# CHAPTER II

# MATERIALS AND METHODS

## A. MATERIALS

### 1. Antiplasmodial Activity of Indonesian Medicinal Plants

a. Plants & reagents

The parts of the medicinal plants studied here were leaves of *T. diversifolia*, leaves and stems of *C. barbata*, stems of *T. crispa*, stems of *A. flava*, as well as leaves and radixes of *P. cauliflora*.

Other materials used were ethanol 70%, n-hexane, ethyl acetate, and methanol.

b. *P. falciparum* strains and cell lines

FcM-29 Cameroon chloroquine-resistant strain was employed for assessing the antiplasmodial activity, while VERO cells were used for testing the cytotoxic.

c. Antimalarial drug controls & reagents

We used Chloroquine (Sigma), DMSO (Sigma), RPMI-1640 (Biowest, France), HEPES, L-glutamine, $AB^+$ human serum (French blood bank, EFS, Toulouse, France), O-type human RBC (French blood bank, EFS, Toulouse, France), $^3$[H] hypoxanthine (Perkin Elmer, France), and complete medium (MEM, FCS, L-glutamine, penicillin, streptomycin, NEAA 1x).

d. Tools

The tools employed were maceration chamber, water bath, rotary evaporator, glass instruments, conical tubes, centrifuge, vortex, freezer, refrigerator, $CO_2$ incubator, 96-well plates, cell counter (Perkin Elmer) and Class II Biosafety cabinet.

## 2. Antiplasmodial Activity of Gold (I) Complexes

a. Tested compounds

Twenty-five synthesized compounds were tested in this study consist of the imidazolium salts (proligands: compunds **1** – **9**, and **11**; zwitterionic proligand: compound **10**) and the mononuclear gold (I) bis(NHC) complexes (compound **12** – **25**).

All these compounds were obtained from group V UPR 8241 LCC-CNRS, Toulouse, France.

b. *Plasmodium falciparum* strains and cell lines

Chloroquine-resistant *P. falciparum* FcM-29 Cameroon strain was used for assessing the antiplasmodial activity while VERO cells were used for testing the cytotoxic.

c. Antimalarial drugs and reagents

The materials used in this study were Chloroquine (Sigma), artemisinin (TCI), DMSO (Sigma), RPMI-1640 (Biowest, France), HEPES, L-glutamine, $AB^+$ human serum (French blood bank, EFS, Toulouse, France), O-type human RBC (French blood bank, EFS, Toulouse, France), $^3$[H] hypoxanthine (Perkin Elmer, France), and complete medium (MEM, FCS, L-glutamine, penicillin, streptomycin, NEAA 1x).

d. Tools

The tools utilized were water bath, rotary evaporator, glass instruments, conical tubes, centrifuge, vortex, freezer, refrigerator, $CO_2$ incubator, 96-well plates, cell counter (Perkin Elmer) and Class II Biosafety cabinet.

## 3. K13 Gene and Artemisinin Resistance

a. Strains & reagents

The *P. falciparum* strains used in this research were *P. falciparum* clinical isolates from Cambodia (Cam3.I, Cam3.II, Cam5, Cam2, CamWT) and reference laboratory lines (F32-TEM, F32-ART, V1/S, FCB, Dd2) provided by four different laboratories. In our laboratory, we conducted the research using FCB, Dd2, and F32 strains.

Other materials used were RPMI-1640 (Biowest, France), HEPES, L-glutamine, $AB^+$ human serum (French blood bank, EFS, Toulouse, France), O type human RBC (French blood bank, EFS, Toulouse, France), Dihydroartemisinin (TCI), DMSO (Sigma), Percoll® (Sigma-Aldrich), PBS, Heparin, D-sorbitol (Sigma-Aldrich), immersion oil, and Giemsa stain (Diff quick®).

b. Materials

The tools utilized were Class II biosafety cabinet, $CO_2$ incubator, water-bath, scale, pipette and tips, flask culture, centrifuge, conical tube, filter 0.22µM pore size, 48-well plates, glass slides, microscope, and counter.

## 4. Dihydroartemisinin-Piperaquine Resistance in Kupang

a. Materials

The materials used were dihydroartemisinin-piperaquine tablets (Darplex), dihydroartemisinin (TCI), RPMI-1640 (Sigma), ACD anticoagulant, O type RBC, culture medium (RPMI-1640, HEPES, Gentamycin, Hypoxanthine, Albumax II, heat-inactivated human serum), DMSO (Sigma), immersion oil, Giemsa stain (Merck), syringe (Terumo), filter paper (Whatmann), K13-propeller primers,

dNTP (Biodynes), $MgCl_2$, *Taq* DNA polymerase (Solis Biodyne), and ethidium bromide stain.

b. Strains

The *P. falciparum* strain employed in this study was *P. falciparum* isolates from malaria patients in Kupang, East Nusa Tenggara.

c. Tools

The tools involved were conical tubes, glass instruments, glass slides, microscope, counter, 48-well plates, $CO_2$ incubator, candle jar, Class II biosafety cabinet, water bath, balance, pipette and tips, Duran® bottles, flask cultures, centrifuge, and filters 0.22μM pore size.

## B. METHODS

### 1. Antiplasmodial Activity of Indonesian Medicinal Plants

a. Plant collections and identification

*Tithonia diversifolia* (Asteraceae; voucher specimen 007-02/L-PB/UII/2013) was collected in Sleman District of Yogyakarta, and *Cyclea barbata* (Menispermaceae; voucher specimen 008-03/L.S-PB/UII/2013) was gathered in Bantul District of Yogyakarta, while *Tinospora crispa* (Menispermaceae; voucher specimen 009-03/S-PB/UII/2013) was obtained in Yogyakarta City. Meanwhile *Arcangelisia flava* (Menispermaceae; voucher specimen 010-04/S-PB/UII/2013) was gathered in South Borneo, and *Pycnarrhena cauliflora* (Menispermaceae; voucher specimen 011-01/S.R-PB/UII/2013) was collected in Sintang District, West Borneo.

The plants were identified in the Laboratory of Biology, Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia (Herbarium of Laboratory of Biology, Department of Pharmacy, Faculty of Science and Mathematics, UII, Yogyakarta, Indonesia), and their samples were air-dried (50-60ºC) and powdered.

b. Plant extraction

Similar to the traditional ways, the parts of the plant taken for the extraction included leaves of *T. diversifolia*, stem and leaves of *C. barbata*, stem of *T. crispa*, stem of *A. flava*, as well as stem and radix of *P. cauliflora.*

Powdered samples of *T. diversifolia* (leaves), *C. barbata* (stem and leaves), *T. crispa* (stem), and *A. flava* (stem) were extracted by maceration in 70 % ethanol for 24 hours, 300 g of plant powder with 1.5 L of solvent. This process was repeated for two days consecutively using fresh 70% ethanol. Following 3 days re-maceration in ethanol, the extracts were filtered and the filtrates evaporated using a rotary vacuum evaporator, to attain ethanol extract. Some of the ethanol extract were then fractionated by n-hexane by liquid-liquid separation method which create two separate layers (n-hexane and first ethanol layer). The n-hexane fraction was acquired after the n-hexane layer was evaporated. The fractionation process was continued by fractionated the ethanol layer using ethyl acetate (liquid-liquid separation) which produce ethyl acetate and second ethanol layer. Both layer was then evaporated separately yielded ethyl acetate fraction from ethyl acetate layer and fraction residue from second ethanol layer, in case there were active compounds left behind.

Contrary to previous plants, radix part of *P. cauliflora* was extracted by n-hexane to attract the lipophilic compound from the plant. The filtrate then evaporated and become n-hexane extract. Meanwhile, the precipitate of n-hexane extraction was further extracted using dichloromethane to obtain dichloromethane extract. Then, the precipitate from previous dichloromethane extraction was employed for methanol extraction. The methanol extract was gained from the evaporation of the filtrates.

At the end of extraction process, from each part of leaves of *T. diversifolia,* stem and leaves *C. barbata*, and stem of *T. crispa* were gained ethanol extract, n-hexane fraction, ethyl acetate fraction, and fraction residue. Meanwhile, from stem of *A. flava* there were ethanol extract, n-hexane fraction and ethyl acetate fraction. Yet, for each part of stem and radix of *P. cauliflora* produce n-hexane, dichloromethane and methanol extracts. Finally, it yielded 25 extracts and fraction that ready for investigation its antiplasmodial activities.

c. Reagent preparation

To evaluate the antiplasmodial activities, the stock solutions of the extracts (1 mg/mL) were prepared in dimethylsulfoxide (DMSO, Sigma, France) and then diluted in RPMI 1640 (Biowest, France). The dilutions were checked to confirm that the extracts did not re-precipitate under these conditions. The extracts were then tested immediately after their dissolution in DMSO and RPMI, kept at 4°C for 3-8 days, and re-tested. The residual DMSO in the assay was 2% and this was evaluated individually to check that this solvent percentage had no activity on parasite cultures. The extract concentrations for both chemo-sensitivity and cytotoxic assays were 0.1µg/mL, 1µg/mL, 10µg/mL, and 50µg/mL.

d. Chemo-sensitivity assay

The antiplasmodial activity was evaluated with the FcM29-Cameroon chloroquine-resistant *Plasmodium* strain and cultured continuously according to the Trager and Jensen method [192], in a 5% $CO_2$ atmosphere at 37°C by considering the modifications previously reported [193]. In brief, the parasites were kept *in vitro* in human red blood cells and diluted in RPMI 1640 medium, added by 25 mM HEPES, L-Glutamine and completed with 5% human serum (French blood bank, EFS, Toulouse, France). The antiplasmodial activity was assessed similarly to what was previously reported by Desjardins et al. [194] and modified as follows. Extract dilutions and reference compounds were tested in triplicate for at least 3 times independently, in 96-well plates with cultures at 1% parasitaemia and 1% haematocrit. For each test, the plates of parasite culture were incubated with extracts or reference compounds for 48 h and $^{3}$[H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of incubation [193]. The parasite growth was estimated from [$^{3}$H]-hypoxanthine incorporation. The control parasite culture (without drug and with 2% DMSO) was referred to as 100% growth. The $IC_{50}$ values (50 % inhibitory concentration) were graphically determined as concentrations versus percentage of parasite growth.

e. Cytotoxic evaluation on macrophage and VERO cell lines

The cytotoxicity tests were evaluated on macrophages and VERO cell lines for compounds showing the best anti-leishmanial and antiplasmodial activities, respectively. The evaluation of cytotoxicity by MTT assay on the J774A.1 cell line (mouse macrophage cell line, Sigma-Aldrich) was performed according to Mosmann [195] with slight modifications, and cytotoxicity on VERO cells

(monkey epithelial cell line, Sigma-Aldrich) was assessed using [$^3$H]-hypoxanthine incorporation. In short, cells ($5.10^4$ cells/mL) in 100 µL of complete medium were seeded into each well of 96-well plates and incubated at 37°C in a humidified 5 % $CO_2$ with 95 % air atmosphere. The complete medium consisting of RPMI 1640 was supplemented with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) for J774A.1 cell line and MEM with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), NEAA 1X for VERO cell line. After 24 h incubation, 100 µL medium with various product concentrations and appropriate controls (DMSO and doxorubicin) were added and the plates were incubated for 72 h at 37°C, 5 % $CO_2$. For cytotoxic evaluation on macrophages, each plate well was then microscope-examined to detect any precipitate formation before the medium was aspirated from the wells. One hundred µL of MTT solution (0.5 mg/mL in RPMI) was then added to each well and the cells were incubated for 2 h at 37°C. Then, the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting formazan crystals. The plates were shaken vigorously for 5 min and absorbance was measured at 570 nm with a microplate spectrophotometer (EON).

For cytotoxic evaluation on VERO cells, the plates were incubated with the test compounds for 48 h and [$^3$H]-hypoxanthine (Perkin Elmer, France) added to the medium 24 h after the beginning of the incubation [193] to estimate VERO cell growth. The control VERO cell culture (drug-free but with 2 % DMSO) was referred to as 100 % of growth. The $IC_{50}$ values (50 % inhibitory concentration) were graphically determined as concentrations *versus* percentage of parasite growth.

f. Anti-*Babesia* activity

*Babesia divergens* (Rouen 1986 strain) was cultured continuously according to Précigout *et al* [196]. The parasites were maintained *in vitro* in human red blood cells ($O^{\pm}$; EFS; Toulouse, France), diluted to 6 % haematocrit in RPMI 1640 medium (Biowest, France) supplemented with 30 nM $NaHCO_3$ and complemented with 10 % human $AB^+$ serum (EFS).

The antibabesial activity of extracts was evaluated by the same radioactive micromethod as that for *Plasmodium* in 96-well culture plates except for the incubation time, which was reduced to 24 h. The drug-free control parasite cultures were considered as 100 % growth, and an imidocarb sensitivity control was included in every plate. $IC_{50}$ was determined graphically from concentration *versus* percentage of inhibition curves.

g. Anti-leishmanial activity on promastigote state

The effect of the compounds tested on the growth of *Leishmania infantum* (line MHOM/MA/67/ITMAP-263) promastigotes was assessed by Luciferase Assay. Briefly, promastigotes in log-phase in RPMI 1640 medium (Biowest, France) were supplemented with 10 % fetal calf serum (Hyclone, ThermoFischer, France), 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL geneticin (Sigma, France)) were incubated at an average density of $10^6$ parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO or MeOH (final concentration less than 0.5 % v/v), in duplicate. Appropriate controls treated by DMSO, MeOH and amphotericin B (reference drug purchased from Sigma Aldrich) were added to each set of experiments. After a 72h incubation period at 24°C, each plate well

was then microscope-examined for any possible precipitate formation. To estimate the luciferase activity of promastigotes, 80 µl of each well was transferred to white 96-well plates, Steady Glow reagent (Promega) was added according to the manufacturer's instructions, and the plates were incubated for 2 min. Luminescence was measured in a Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50 % ($IC_{50}$) is defined as the concentration of drug required to inhibit 50 % of the metabolic activity of *Leishmania infantum* promastigotes compared to the control. $IC_{50}$ was calculated by non-linear regression analysis processed on dose–response curves, using TableCurve 2D V5® software. $IC_{50}$ values are the geometric mean values calculated from three independent experiments.

**2. Antiplasmodial Activity of Gold (I) Complexes**

a. Synthesized compounds

All the synthesized compounds (the imidazolium salts and the mononuclear gold (I)-bis(NHC) complexes) have been prepared in powders and ready to be tested.

b. Reagent preparation

To evaluate the antiplasmodial activities, the stock solutions of the synthesized compounds (1 mg/mL) were prepared in dimethylsulfoxide (DMSO, Sigma, France) and then diluted in RPMI 1640 (Biowest, France). The dilutions were checked to confirm that the synthesized compounds did not re-precipitate under these conditions. The synthesized compounds were then tested immediately after their dissolution in DMSO and RPMI, kept at 4°C for 3-8 days, and re-tested. The residual DMSO in the assay was 2% and this was evaluated individually to

check that this solvent percentage had no activity on parasite cultures. The synthesized compounds concentrations for both antiplasmodial and cytotoxic assay were 0.1µg/mL, 1µg/mL, 10µg/mL, and 50µg/mL.

c. Antiplasmodial assay

The antiplasmodial activity was examined for the chloroquine-resistant strain FcM29-Cameroon and cultured continuously using to Trager and Jensen method [192], in a 5% $CO_2$ atmosphere at 37°C, and considering the modifications previously reported [193]. In brief, the parasites were kept *in vitro* in human red blood cells and diluted in RPMI 1640 medium, added by 25 mM HEPES, L-Glutamine and completed with 5% human serum (French Blood Bank, EFS, Toulouse, France). The antiplasmodial activity was assessed similarly to what was previously reported by Desjardins *et al.* [194] and modified as follows. Synthesized and reference compounds were tested in triplicate at least 3 times independently, in 96-well plates with cultures at 1% parasitaemia and 1% haematocrit. For each test, the plates of parasite culture were incubated with synthesized or reference compounds for 48 h and $^{3}$[H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of incubation [193]. The parasite growth was estimated from [$^{3}$H]-hypoxanthine incorporation. The control parasite culture (without drug and with 2% DMSO) was referred to as 100% growth. The $IC_{50}$ values (50% inhibitory concentration) were graphically determined as concentrations versus percentage of parasite growth.

d. Cytotoxic evaluation on VERO cell lines

The cytotoxicity tests were performed on VERO cell lines for compounds showing the best antiplasmodial activities. The evaluation of cytotoxicity on VERO cells (monkey epithelial cell line, Sigma-Aldrich) was assessed using [$^{3}$H]-

hypoxanthine incorporation. In short, cells ($5.10^4$ cells/mL) in 100 µL of complete medium were seeded into each well of 96-well plates and incubated at 37°C in a humidified 5% $CO_2$ with 95% air atmosphere. The complete medium consisting MEM with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), NEAA 1X.

The plates were incubated with the test compounds for 48 h and [$^3$H]-hypoxanthine (Perkin Elmer, France) added to the medium 24 h after the beginning of the incubation [193] to estimate VERO cell growth. The control VERO cell culture (drug-free but with 2% DMSO) was referred to as 100% of growth. The $IC_{50}$ values (50% inhibitory concentration) were graphically determined as concentrations *versus* percentage of cell growth.

**3. K13 Gene and Artemisinin Resistance**

a. Continuous culture of *P. falciparum*

The artemisinin resistance was assessed against various clinical isolates (Cam3.I, Cam3.II, Cam5, Cam2, and CamWT) and laboratory lines (F32 TEM, V1/S, FCB, and Dd2). All strains were cultured continuously according to Trager and Jensen method [192], in a 5% $CO_2$ atmosphere at 37°C, taking into account the modifications previously described [193]. The parasites were briefly maintained *in vitro* in human red blood cells and diluted in RPMI-1640 medium, supplemented with 25 mM HEPES, L-Glutamine and completed with 5% human serum (French Blood Bank, EFS).

b. Reagent preparations

1) Stock and test solution of Dihydroartemisinin

Dihydroartemisinin (DHA) stock solutions were prepared in DMSO to obtain 1mg/mL of stock solution. To have a 200μg/mL (700nM) of stock solution, the initial stock solution 1mg/mL was diluted 5-fold in DMSO. A total of 50 μL aliquots of 700nM stock solution was stored in sterile 1.5 mL micro centrifuge tubes at -20°C. For DHA test solution, 20 μL of DHA 700nM stock solution was added to 2 mL of culture medium.

2) Control solution of Dimethylsulfoxide (DMSO)

Dimethylsulfoxide (DMSO) control solution was prepared by adding 20 μL of DMSO to 2 mL of culture medium.

3) Percoll® solutions

Percoll® solutions were prepared at room temperature, in a Class II biosafety cabinet, using sterile reagents. Nine volumes of Percoll® (Sigma-Aldrich) were mixed with one volume of 10x PBS to make a Percoll® 90% solution (stored at 4°C, up to 2 months). For RSA assay, 7.5 volumes of Percoll® 90% solution were mixed with 1.5 volumes of heparinized RPMI-1640 (freshly prepared 15 μL heparin mixed with 10 mL RPMI-1640) to obtain a Percoll® 75 % solution prepared directly prior to the assay.

4) Sorbitol solution

A total of 50 grams of D-sorbitol (Sigma-Aldrich) was dissolved in 1L of distilled water resulting in a 5% D-Sorbitol solution. Using a 0.22 μM filter, the homogenized solution was sterilized and stored at 4°C (up to 1 year).

c. *In vitro* RSA assay

These assays were carried out as previously described [197], with minor modifications. In summary, 10-15 mL parasite cultures were synchronized 1-2 times using 5% sorbitol (Sigma-Aldrich). Synchronous multinucleated schizonts were incubated in RPMI-1640 containing 15 units/mL sodium heparin for 15 min at 37°C to disrupt agglutinated erythrocytes, concentrated over a gradient of 75% Percoll® (Sigma-Aldrich), washed once in RPMI-1640, and incubated for 3 hours with fresh erythrocytes to allow time for merozoite invasion. Cultures were then subjected again to sorbitol treatment to eliminate remaining schizonts. The 0-3 hours of post-invasion rings were adjusted to 1% parasitemia and 2 % hematocrit in 1 mL volume (in 48-well plates) and exposed to 700nM DHA or 0.1% DMSO (solvent control) for 6 hours. Duplicate wells were established for each parasite line ± drug. As much as 1 mL of the cultures was then transferred to 15 mL conical tubes and centrifuged at 1600 rpm for 5 min to pellet the cells, and the supernatants were carefully removed. As a washing step to remove drug, 9 mL of culture medium was added to each tube, the cells were re-suspended and centrifuged, and the medium was aspirated. Fresh lacking drug medium was then added to the cultures, and this mixture was returned to standard culture conditions for 66 hours.

Parasite viability was assessed though microscopic examination of Giemsa-stained thin blood smears by counting the parasites developed into second-generation rings or trophozoites with normal morphology. To obtain a homogenous smear for all slides, the culture was re-suspended, transferred into an Eppendorf tube, and briefly centrifuged (2000 rpm for 30 seconds). Exactly 2 μL of the pellet was then used for each smear. Parasitemias were calculated from a

total of at least 40,000 erythrocytes per assay. Slides were read from the two duplicate wells per assay by two separate microscopists, each of them was examined for at least 10,000 erythrocytes per slide. In the case of more than 20% discrepancy in parasite counts, the slides were further examined by a third microscopist. The percentage of survival was calculated as the parasitemia in the drug-treated sample divided by the parasitemia in the untreated sample multiplied by 100.

$$\%\ \text{survival} = \frac{\text{drug treated sample parasitemia (DHA wells)}}{\text{untreated sample parasitemia (DMSO wells)}} \times 100$$

The artemisinin resistance was described as ≥ 1.5% of survival percentage.

**4. Dihydroartemisinin-Piperaquine (DHP) Resistance in Kupang**

a. Ethics approval submission

The study protocol and informed consent have been approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Gadjah Mada University, Yogyakarta (ref number KE/FK/222/EC). Therefore, the entire process of the study has been conducted under ethical considerations.

b. Study population

The population of this study covered several areas in Kupang East Nusa Tenggara which has a high case incidence (HCI) based on the Annual Parasite Incidence (API). The samples followed the inclusion criteria, such as patients diagnosed for non-complicated malaria caused by particularly *P. falciparum* (slide-confirmed malaria), those having fever or history of fever 48 h prior to presentation, patients seeking treatment in primary health center or district referral

hospital, and those agreeing to sign the informed consent and follow the study. The exclusion criteria were pregnant or lactating women, children under 5 kg, anyone already taking malaria treatment for 4 weeks beforehand, diagnosed for complicated malaria (co-infection), infected by other *Plasmodium* species than *P. falciparum*, having signs of severe malaria, severe malnutrition, serious underlying disease (renal, cardiac, or hepatic), or known to be allergic to the research drugs.

c. Drug administration and follow-up

The dihydroartemisinin-piperaquine (DHP) was administered 3 times daily for 3 days with a dosage of 2-4 mg/kg of body weight (BW) of dihydroartemisinin and 16-32 mg/kg BW of piperaquine. Subsequent follow up appointments were scheduled for days 2, 3, 7, 14, 21, 28, 35, and 42; in addition, the blood smear was examined during the follow-up periods and collected on filter paper for DNA extraction. During the follow-up visits, patients were asked about the general condition of well-being as well as open questions about possible adverse effects that might occur since the last visit and examined by the research physicians.

d. Parasite isolate preparation

Venous blood was collected aseptically into an ACD anticoagulant tube from the patients before a treatment administration ($H_0$). Using a drop of blood, parasitemia evaluation was performed by a Giemsa-stained thin blood smear. Following parasitemia determination, blood centrifugation (800g for 5 minutes) separated RBC pellet from the plasma and buffy coat. The RBC pellet was then

washed using RPMI (20 mL of RPMI, centrifugation at 800 g for 5 minutes and removal of supernatant, consecutively) for 6-8 times, preparing infected RBCs to be processed for *ex-vivo* RSA.

e. The *ex-vivo* Ring-stage Survival Assay (RSA)

The *ex-vivo* Ring-stage Survival Assay (*ex-vivo* RSA) was performed on the parasite isolates freshly collected from patients with uncomplicated *P. falciparum* malaria. The parasites were exposed to 700nM dihydroartemisinin (DHA) for 6 hours – approximating their drug exposure in patients treated with artemisinin – and their survival was assessed 72 hours later [198].

To perform the *ex-vivo* RSA, ≥ 0.5% parasitemia was required. The parasite solution was prepared by adding 50µL of infected RBCs to 2.5 mL of culture medium. If the parasitemia was > 1%, pellet was diluted with uninfected RBCs to obtain 0.5-1% parasitemia and with culture medium to obtain 2% hematocrit. A thin smear was made for determining initial parasitemia. The culture medium consisted of RPMI-1640 medium, HEPES, gentamicin, hypoxanthine, Albumax II, and heat-inactivated human serum.

In a 48-well culture plate, 900 µL of parasite solution was transferred to each well and added with 100 µL of DMSO solution in the control well or 100 µL of DHA solution (700nM) in the treatment well. The 48-well culture plate was maintained in an incubator under $37^0$C, humid atmosphere, 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for exactly 6 hours. After 6 hours, the contents of each well were transferred into 15mL centrifuge tubes for a following centrifugation at 800g for 2 minutes. Then, 12 mL of pre-warmed RPMI was added for washing the RBCs pellet prior to a transfer into new wells in the 48-well culture plate with 1 mL of

culture medium to resuspend the pellet. The new 48-well culture plate was maintained in an incubator ($37^0$C, humid atmosphere, 5% $O_2$, 5% $CO_2$, and 90% $N_2$) for 66 hours. Finally, the Giemsa-stained thin smear of each well was made at the end of incubation and determined for the percentage of survival under a microscope examination.

At 100x magnification in immersion oil, the number of infected RBCs containing viable parasites were counted in a total of 10,000 RBCs. Only viable parasites were scored; so, vacuolated and pyknotic forms were not considered. To determine the proportion of viable parasite (% survival), the parasitemia of DHA exposed was compared to the parasitemia of non-exposed (DMSO).

The *ex-vivo* RSA would be performed also at any days of treatment failure emerging during the follow-up.

f. Sequencing the K13-propeller domain

The molecular marker the *PfK13*-propeller domain was amplified using the following primers: for the primary PCR (K13-15'-cggagtgaccaaatctggga-3' and K13-4 5'-gggaatctggtggtaacagc-3') and the nested PCR (K13-2 5'-gccaagctgccattcatttg-3' and K13-3 5'-gccttgttgaaagaagcaga -3'), one µL of DNA was amplified with 1µM of each primer, 0.2 mM dNTP (Solis Biodyne), 3 mM $MgCl_2$ and 2U *Taq* DNA polymerase (Solis Biodyne), using the following cycling program: 5min at 94ºC, then 40 cycles of 30 s at 94ºC, 90 s at 60ºC, 90 s at 72ºC and final extension of 10 min at 72ºC. For the nested PCR, 2 µL of primary PCR products was amplified under the same conditions, except for the $MgCl_2$ concentration (2.5 mM). The PCR products were detected using 2% agarose gel electrophoresis and ethidium bromide staining. Meanwhile, the double-strand

sequencing of PCR products was performed using Macrogen, and the sequences were then analysed using MEGA5 software version 5.10 to identify specific SNP combinations.

# CHAPTER III

# RESULTS

This chapter points out various strategies investigated for malaria elimination, particularly in Indonesia. The first part is dedicated to discover new antimalarial drugs either from Indonesian medicinal plants or with synthesis of organometallic compounds. Moreover, as artemisinin resistance has emerged in South-East Asia, it is important to confirm the major role of molecular markers involved in this resistance. Defined by reverse genetic of the mutation involvement of *Pfk13* gene in this resistance, the study of its spread in South East Asia regions is used to monitor the possible appearance of artemisinin resistance in malaria endemic areas in Indonesia.

## A. Antiplasmodial Activity of Indonesian Medicinal Plants

1. Results obtained

The emerging resistance of artemisinin, the basis of recent first-line combination therapy, has threatened the global effort to reduce malaria caused by *P. falciparum*. Moreover, there are no current antimalarial drug to replace artemisinin, hence discovering new antimalarial drug is inevitable. One of the strategies to find new antimalarial agents is the *in vitro* evaluation of crude extracts activities of medicinal plants against *P. falciparum* strains. Indonesia's rich biodiversity features medicinal plants used traditionally for a variety of disease treatments, including malaria. In cooperation with CNRS - LCC in Toulouse, France, the Faculty of Medicine of Universitas Gadjah Mada conducted the assessment of antiplasmodial activities of five medicinal plants: *T. diversifolia, C. barbata, T. crispa, A. flava* and *P. cauliflora*. These plants were selected due to their popularity as a fever therapy among Indonesians and because they naturally grow in Indonesia. In addition, some of these plants possess not only medicinal

advantages but also economic benefits, such as *C. barbata* prepared for drinks and sold in sidewalks. In contrast, some of them offer limited information on their medicinal and economic benefits, such as *P. cauliflora,* making people ignore their cultivation and the plants become endangered.

This France-Indonesia collaboration aimed to conduct a standardized study targeting antiplasmodial activities of these extracts. In addition, since *Plasmodium* has similar biochemical pathways with *Babesia*, another apicomplex parasite, we intended to discover shared active compounds on these two close pathogens. Moreover, as malaria patients can also be co-infected by leishmaniasis in areas where the two parasites coexist, especially in tropical and subtropical countries such as in South America as one of the endemic regions [9], it was also interesting to assess the antileishlaniasis activities of the selected extracts. This study was completed by an assessment of the cytotoxicity on mammalian cells to determine the specificity of their activities.

Our team gathered the plants from different areas in Indonesia and identified them in the Biology Laboratory of the Pharmacy Department of Universitas Islam Indonesia in Yogyakarta. A maceration process with various solvent polarities was utilized to extract the active compounds of the plants. The chloroquine-resistant strain of *P. falciparum* FcM29-Cameroon was selected to evaluate the antiplasmodial activity of these various extracts according to Desjardins's method with slight modifications. The same assay method was used for anti-babesial activity, except that *B. divergens* was cultured following the method by Précigout *et al* [196]. The anti-leishmanial activity was examined by a luminescent assay based on luciferase activity on *L. infantum* blood stage preceding the macrophage invasion (promastigotes). For the extracts with the best antiplasmodial and anti-leishmanial activities, cytotoxicity tests were conducted against macrophages and VERO cell lines using MTT assay and [$^{3}$H]-hypoxanthine incorporation, respectively.

The results figured out that, among the 25 crude extracts and fractions, seven indicated promising antiplasmodial activities ($IC_{50}$ < 5µg/mL) [135]: *T. diversifolia* ethanol extract and ethyl acetate fraction, *A. flava* ethanol extract, N-hexane and ethyl acetate fractions, *P. cauliflora* radix part dichloromethane, and methanol extracts. Additionally, these best antiplasmodial extracts were then tested on the VERO cell line to investigate the specificity of their activity which was then translated into selectivity index (SI) as a ratio between cytotoxicity and activity (antiplasmodial activity, in this regard). However, only *A. flava* and *P. cauliflora* radix dichloromethane extracts featured high selectivity against VERO cell lines (> 50µg/mL).

*Tithonia diversifolia* or locally known as kembang bulan was active against *P. falciparum* (ethanol extract and ethyl acetate fraction), but their cytotoxic values were similar, with less than 1 selectivity index. This low selectivity index (SI) indicated that this plant offers limited interest for further investigation although several previous studies demonstrated its activity. The ether extract of aerial parts showed *in vitro* antiplasmodial activity ($IC_{50}$: 0.75 µg/mL against CQ-sensitive strain FCA) which led to extraction of active compound, tagitinin C, a sesquiterpene lactone possessing 0.33 µg/mL $IC_{50}$ against FCA [140]. Not only *in vitro* activity, *T. diversifolia* was also reported on its *in vivo* antiplasmodial activity in a mouse malaria model at concentration of 200 mg/kg [141].

Meanwhile, with the $IC_{50}$ around 3 µg/mL, *A. flava* (also known as kayu kuning) ethanol extract, n-hexane and ethyl acetate fractions displayed a good antiplasmodial activity. In addition, their cytotoxicity against VERO cell showed high selectivity level (SI: >50). Based on literature, the antiplasmodial activity of *A. flava* extract has been published with the range of $IC_{50}$ from 0.4 to 8.6 µg/mL against *P. falciparum* [166] and one of its active compounds, berberine, has been proved to inhibit telomerase activity of *P. falciparum* [167]. The result of

this study supports those previous findings with additional information about selectivity of the extracts.

Sengkubak or *P. cauliflora*, a medicinal plant that has not been widely explored, demonstrated the best $IC_{50}$ values on its dichloromethane and methanol extracts, especially from radix part (3.3 µg/mL for both solvents) even though the high selectivity index only showed for the radix dichloromethane extract (SI:>15). This plant was reported having antioxidant [171] and cytotoxic activity [170] with bisbenzylisoquinoline alkaloids (obaberine and pycnarrhine) as active compounds [169]. Yet, no study has revealed its antiplasmodial activity to date. This plant then appears particularly promising and deserves further study since its active compound, bisbenzylisoquinoline alkaloids, has been reported to have antiplasmodial activity [199].

Unexpectedly, even though it is commonly used for traditional malaria treatment, extract and fraction from *C. barbata* (stem and leaf) or *T. crispa* (stem) did not show antiplasmodial activity. After 3 independent experiments, its $IC_{50}$ values were constantly beyond 5 µg/mL, a threshold to classify good activity of the extract. Previous studies found that bisbenzylisoquinoline alkaloids (tetrandrine, limacine, thalrugosine, homoaromaline, and cycleapeltine extracted from the root of *C. barbata* have both cytotoxic and antimalarial activities [200]. Other report also stated that those bisbenzylisoquinoline alkaloids demonstrated high antiplasmodial activity [201, 202]. On the other hand, a study on *T. crispa* reported its antiplasmodial activity of the stem against *P. berghei* [203] and *in vitro* activity from methanol stem extract with the $IC_{50}$ value of 0.8 mg/mL [159]. That $IC_{50}$ value correspond to the result of this study, which indicate low interest in pharmacological activity.

Eight extracts also displayed anti-babesial activities with $IC_{50}$ values ranging from 1.2 to 14.1 µg/mL, with 3 of them close to the control babesial inhibitor, imidocarb (0.5µg/mL).

With a good selectivity index *A. flava* extract and fractions showed that an antiplasmodial activity can also be coupled with an anti-babesial activity.

Concerning the extract assays on *L. infantum*, only 3 had $IC_{50}$ values less than 50µg/mL: the ethyl acetate fraction of *T. diversifolia,* and, dichloromethane and methanol fractions of *P. cauliflora* radix ($IC_{50}$ 21.9, 1.7 and 4.4 µg/mL, respectively). The results showed that *P. cauliflora* radix methanol extract was active against *P. falciparum, Babesia* and *L. infantum* at similar concentrations and relatively low indexes of selectivity, suggesting a global cytotoxic effect in contrast to the dichloromethane fraction at which the cytotoxicity was low on VERO cells.

In conclusion, extracts or fraction with a good antiplasmodial activity can be positively correlated to a good and specific antibabesial activity. Among the most active extracts against *P. falciparum,* only dichloromethane and methanol extract of *P. cauliflora* were active against *L. infantum*. The specific anti-parasite activities of some extracts were confirmed by their weak cytotoxicity on VERO cell line. These particular extracts need an in-depth phytochemical assessment in order to identify and characterize the active compounds either to standardize plant preparations or to chemically develop new anti-parasitic compounds as detailed in the article below submitted for publication.

2. Corresponding publication

ARTICLE IN PRESS

Journal of Herbal Medicine xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Herbal Medicine

journal homepage: www.elsevier.com/locate/hermed

Research paper

# Antiprotozoal properties of Indonesian medicinal plant extracts

Arba P. Ramadani[a,b,c,d], Lucie Paloque[a,b], Hugo Belda[a,b,e], Hady Anshory Tamhid[d], Masriani[f], Jumina[g], Jean-Michel Augereau[a,b], Alexis Valentin[b,e], Mahardika Agus Wijayanti[h], Mustofa[c,*], Françoise Benoit-Vical[a,b,**]

[a] CNRS, LCC (Laboratoire de Chimie de Coordination) UPR8241, F-31077 Toulouse Cedex 4, France
[b] Université de Toulouse, UPS, INPT, F-31077 Toulouse Cedex 4, France
[c] Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
[d] Department of Pharmacy, Faculty of Science & Mathematics, Islamic University of Indonesia, Yogyakarta, Indonesia
[e] UMR 152 Pharma-DEV IRD-UPS, Toulouse, France
[f] Study Program Chemistry of Education, Department of Mathematics and Natural Science Education, Tanjungpura University, Pontianak, Indonesia
[g] Department of Chemistry, Faculty of Mathematics and Natural Science, Gadjah Mada University, Sekip Utara, Yogyakarta, Indonesia
[h] Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

ARTICLE INFO

*Keywords:*
*Plasmodium falciparum*
Traditional medicine
Indonesia
Antiparasitic drugs

ABSTRACT

*Tithonia diversifolia, Cyclea barbata, Tinospora crispa, Arcangelisia flava, Pycnarrhena cauliflora* are plants used in Indonesia for the traditional treatment of malaria. In the search for new antiparasitic drugs, the parts traditionally used of these 5 plants were extracted with ethanol and then fractionated with various solvents and evaluated *in vitro* against *Plasmodium falciparum* and also against *Babesia divergens* and *Leishmania infantum*. Seven crude plant extracts out of 25 tested displayed high antimalarial activities with $IC_{50} < 5$ µg/ml and in the cases of some of them an interesting selectivity regarding their cytotoxicity against mammalian cells. *A. flava* appeared to be the most promising antiplasmodial plant with the highest antiplasmodial activity ($IC_{50}$ values less than 3 µg/ml) and the weakest cytotoxicity. By contrast, only *P. cauliflora* radix, through its dichloromethane and methanol fractions also demonstrated a high activity against *L. infantum*, with $IC_{50}$ values around 3 µg/ml; their high selectivity index, especially on VERO cells, hypothesises a specific parasiticidal action. Moreover, for all the extracts showing antiplasmodial activity, a positive correlation was demonstrated with antibabesial activity, suggesting that these antiplasmodial extracts could be a potential source of antibabesial compounds. These preliminary results confirm the antiplasmodial interest of some of these plants used in traditional medicine but also their effects on leishmaniasis and babesiosis. Ongoing phytochemical investigations should allow identification of the chemical series responsible for these activities.

## 1. Introduction

Malaria is the most prevalent parasitic disease in the world and continues to be one of the largest public health problems, especially in the developing countries. There is an estimated 3.2 billion people at risk of being infected and leading to an estimated 584,000 malaria deaths 78 % of which concern children under 5 years (WHO, 2014c). *Plasmodium falciparum*, the most deadly species of malaria parasite already shows resistance to all antimalarial drugs, including artemisinin and its derivatives, which are the latest and the most effective treatments of malaria (Straimer et al., 2015; WHO, 2014c; Witkowski et al., 2009; Wongsrichanalai et al., 2002). Unfortunately the use of artemisinin-based combination therapies (ACTs) advocated by the World Health Organization (WHO) did not allow for the emergence and the spread of multi-resistance in the Greater Mekong region (Cambodia, Vietnam, Thailand, Myanmar and Laos) threatening the strategies of malaria eradication in the other endemic areas (WHO, 2014b). Consequently there is an urgent need to discover new antimalarial agents, and crude extracts of natural products appear to be a promising route to follow as they are more affordable for people in developing countries (Benoit-Vical, 2005; Benoit-Vical et al., 2008, 2003).

Indonesia is rich in medicinal plants which the population use traditionally for curing diseases including malaria. This study, based on ethnobotanical data, was carried out on 5 medicinal plants selected as potential sources of antimalarial agents: *Tithonia diversifolia, Cyclea barbata, Tinospora crispa, Arcangelisia flava, Pycnarrhena cauliflora.*

* Corresponding author at: Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia.
** Corresponding author at: CNRS, LCC (Laboratoire de Chimie de Coordination) UPR8241, 205 route de Narbonne, BP 44099, F-31077 Toulouse Cedex 4, France.
*E-mail addresses:* mustofajogja@yahoo.com (Mustofa), Francoise.Vical@inserm.fr (F. Benoit-Vical).

http://dx.doi.org/10.1016/j.hermed.2017.06.004
Received 23 May 2016; Received in revised form 13 June 2017; Accepted 28 June 2017


Please cite this article as: Ramadani, A.P., Journal of Herbal Medicine (2017), http://dx.doi.org/10.1016/j.hermed.2017.06.004

Except *T. diversifolia,* the 4 other plants are from the Menispermaceae family studied largely for their use in traditional medicine and principally for malaria treatment (Verpoorte et al., 1982b).

Some of these plants have already been investigated for their antiplasmodial activities and pharmacological properties but no standardised studies have yet been performed with them. The infusion of *T. diversifolia* leaves is traditionally used for lowering blood glucose (Miura et al., 2005), flatulence, malaria fever (Njoroge and Bussmann, 2006), and also the healing of wounds. An ethanol extract of *T. diversifolia* exhibited anti-malarial activity by its leaf extracts (Oyewole et al., 2008). *C. barbata* leaves have been used by the Javanese for gastric problems and as a prophylactic against malaria fever (Manilal and Sabu, 1985; Saxena et al., 2003). *T. crispa* is traditionally used for the treatment of fever, rheumatic arthritis, hepatitis, anti-hyperglycaemia, and also malaria by using stem infusions (Pathak et al., 1995; Pushpangadan and Atal, 1984). *T. crispa*, which is abundant in the Philippines, is used freely by the natives under the name of *makabuhay* (meaning "You may live"), as a panacea, especially valuable in malarial fevers (The Southwest School of Botanical Medicine, 1918). Supporting the traditional uses, *T. crispa* scientifically proved as anti-hyperglycaemia (Noor and Ashcroft, 1998) and also both *in vitro* and *in vivo* antimalarial activity (Najib Nik et al., 1999; Rungruang and Boonmars, 2009). *A. flava* is traditionally used as a stem decoction for typhoid fever (Mandia, 1999), hepatitis, gastric disturbance and malaria (Subeki et al., 2005). The anti-hepatotoxicity (Wongbutdee et al., 2003) and anti-malarial activity have been profiled by several studies (Lovin et al., 2012; Nguyen-Pouplin et al., 2007; Vennerstrom and Klayman, 1988; Verpoorte et al., 1982a). *P. cauliflora* is a Dayak tribe folk medicine used for flavouring, flatulence (by placing the soaked leaves on the stomach), fever and malaria (by drinking the leaf infusion). Only local accounts published in Indonesian can be cited for these uses. One study of *P. cauliflora* has just revealed its cytotoxic activities related to apoptosis and cell cycle arrest (Masriani and Adnyana, 2011), but its anti-parasitic properties have not yet been established. Indeed, despite widespread traditional use of *P. cauliflora* reported on malaria treatment in Indonesia, to the best of our knowledge, no studies have been conducted on this plant. Other *Pycnarrhena* species have been studied for their biologically active principles (Siwon et al., 1981b; Verpoorte et al., 1978).

Furthermore, because *Plasmodium* and *Babesia* belong to the Apicomplexa phylum they share many common biochemical pathways. We can therefore hypothesise the possibility of finding common active compounds for both pathogens. *Babesia divergens* babesiosis is a widespread illness transmitted by livestock ticks. This pathogen is the main agent of bovine babesiosis in Europe but can also affect splenectomized humans (Ceci et al., 2014; L'Hostis et al., 1995; Zintl et al., 2014). Infections can occur without producing symptoms, but babesiosis may also be severe and sometimes fatal due to the intraerythrocytic development of the parasite (Melhorn and Schein, 1984). There are effective babesiacides, but imidocarb dipropionate is practically the only drug available on the market and therefore the most widely used. More specific new fast-acting treatments for babesiosis should now be developed (Vial and Gorenflot, 2006).

Leishmaniasis, identified as a "Neglected Tropical Disease" by WHO, is another parasitic disease. The pathogen is endemic in 98 countries and territories, with the number of new cases estimated at 1.3 million per year (WHO, 2014a). Nowadays, malaria and leishmaniasis are responsible for over a million deaths a year and threaten more than 350 million people worldwide, mostly, but not only in tropical and subtropical countries.

Here we offer a standardised study that focuses on the search for new antiparasitic drugs targeting *Plasmodium, Leishmania* and *Babesia*. Indeed, even though the five selected plants are traditionally used for the treatment of malaria in Indonesia, the antiplasmodial activities of their different extracts were not totally investigated. The extracts showing the best antiplasmodial activity were also tested on *Babesia divergens* cultures to investigate another Apicomplexa parasite. Moreover, in some tropical zones, malaria and leishmaniasis infections are largely co-endemic, the extracts were thus tested on both *P. falciparum* and *L. infantum* to evaluate their anti-parasite activities as potential sources of new active compounds. The cytotoxicity of these extracts was assessed in parallel in order to evaluate the specificity of their anti-parasitic activities.

## 2. Materials and methods

### 2.1. Plant material

The plants were identified in the biology laboratory of the Department of Pharmacy, Islamic University of Indonesia, Yogyakarta-Indonesia (Herbarium of Laboratory of Biology, Department of Pharmacy, Faculty of Science and Mathematics, UII, Yogyakarta, Indonesia). Samples were air-dried and powdered.

*Tithonia diversifolia* (Hemsl.) A. Gray. (Asteraceae; voucher specimen 007-02/L-PB/UII/2013) was collected in the Sleman district of Yogyakarta. *T. diversifolia* is a shrub, locally called "kembang bulan".

*Cyclea barbata* Miers (Menispermaceae; voucher specimen 008-03/L.S-PB/UII/2013) was collected in the Bantul district of Yogyakarta and locally known as "Cincau rambat" (Javanese). *C. barbata* can be easily distinguished by its deltoidovate, hispid leaves with acuminate apex, finely mucronate acumen and long male and female inflorescences with dense capitate flowers.

*Tinospora crispa* (L.) (Menispermaceae; voucher specimen 009-03/S-PB/UII/2013) known by the vernacular name of "brotowali", was collected from Yogyakarta. *T. crispa* is a woody tropical liana with shiny green leaves.

*Arcangelisia flava* (L.) Merr. (Menispermaceae; voucher specimen 010-04/S-PB/UII/2013), collected in South Borneo, is a large, woody, glabrous and dioecious liana, up to 20 m long; with a stem of up to 5 cm in diameter and with yellow wood exuding a yellow sap when cut.

*Pycnarrhena cauliflora* (Miers.) Diels (Menispermaceae; voucher specimen 011-01/S.R-PB/UII/2013) was collected in Sintang district, West Borneo. This plant can be found in primary or secondary forests and tend to grow in clusters. From 8 species in the genus *Pycnarrhena,* only 4 (*P. australiana, P. ozantha, P. manilensis, P. longifolia*) have been investigated in any detail.

### 2.2. Preparation of plant extract

For *T. diversifolia* (leaves), *C. barbata* (stem and leaves), *T. crispa* (stem), and *A. flava* (stem), powdered samples were extracted by maceration in 70% ethanol for 24 h, 300 g of plant powder with 1.5 L of solvent (Fig. 1). This process was repeated for two days consecutively using fresh 70% ethanol. Each ethanol extract was filtered and filtrates were evaporated to dryness using a rotary vacuum evaporator. Dried ethanol extracts were solubilized in ethanol to be fractionated by *n*-hexane/by liquid–liquid separation method which created two separate layers (*n*-hexane and first ethanol layer). The *n*-hexane layer gave the *n*-hexane fraction after evaporation. The fractionation process was continued by fractionation of the first ethanol layer using ethyl acetate (liquid–liquid separation) which produced ethyl acetate and second ethanol layers giving respectively after evaporation, the ethyl acetate fraction and ethanol fraction residue.

For *P. cauliflora* powdered radix part (Fig. 2) was directly treated by *n*-hexane to extract lipophilic compounds. After filtration the *n*-hexane extract was evaporated to dryness. The precipitate resulting of *n*-hexane extraction was further put back in suspension/solubilized using dichloromethane to obtain dichloromethane extract after filtration. The new precipitate was submitted to a methanol extraction followed by a filtration and an evaporation of the filtrate.

This extraction method was employed to investigate the possible active compounds responsible for plant activities. Particularly for *P.*

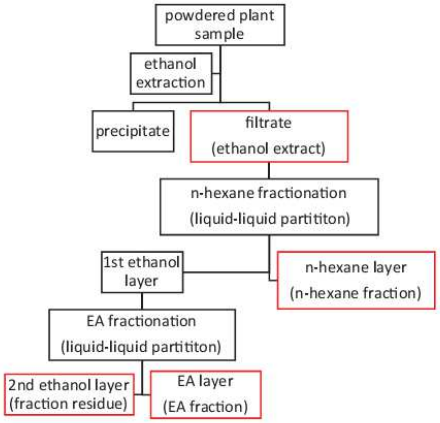



**Fig. 1.** Representative scheme of *T. diversifolia*, *C. barbata*, *T. crispa*, and *A. flava* extraction.
EA: ethyl acetate
Red boxes correspond to extracts used for antiparasitic activity investigations

*cauliflora*, dichloromethane was used to extract active compounds with semi-polar characteristics in order to explore new compounds other than those already published. For each part of leaves of *T. diversifolia*, stem and leaves of *C. barbata*, and stem of *T. crispa* 3 different extracts were obtained: ethanol, *n*-hexane and ethyl acetate fractions, and 1 fraction residue. For stem of *A. flava* only the ethanol, *n*-hexane and ethyl acetate fractions were obtained. For each part of stem and radix of *P. cauliflora* it was produced *n*-hexane, dichloromethane and methanol extracts. Finally, it yielded 25 extracts ready for antiparasitic activity investigations.

To evaluate the anti-parasite (against *Plasmodium*, *Babesia* and *Leishmania*) activities, stock solutions of the 25 crude and semi-purified extracts (1 mg/ml) were prepared in dimethylsulfoxide (DMSO, Sigma, France) and diluted for use in RPMI 1640 (Biowest, France). The dilutions were checked in order to confirm that the extracts did not precipitate under these conditions. The extracts were tested immediately after their dissolution in DMSO and RPMI, then kept at 4 °C for 3–8 days, and tested again to validate the conservation of mother solutions. The maximum percentage of residual DMSO in the assay was 2% and was evaluated alone on parasite cultures to check its absence of activity.

### 2.3. In vitro culture of Plasmodium falciparum and antiplasmodial activity

The antiplasmodial activity was assessed on the FcM29-Cameroon chloroquine-resistant strain of *Plasmodium falciparum*, cultured continuously according to the Trager and Jensen method (Trager and Jensen, 1976), in a 5% $CO_2$ atmosphere at 37 °C, taking into account the modifications previously described (Benoit-Vical et al., 2007). Briefly, the parasites were maintained *in vitro* in human red blood cells and diluted in RPMI 1640 medium, supplemented with 25 mM HEPES, L-glutamine (Biowest, France) and completed with 5 % human serum (French Blood Bank, EFS; Toulouse, France). The antiplasmodial activity was assessed as previously reported by Desjardins et al. (Desjardins et al., 1979) and modified as follows. Extract dilutions and reference compounds were tested in triplicate at least 3 times independently, in 96-well plates with cultures at a parasitaemia of 1 % and a haematocrit of 1 %. For each test, the plates of parasite culture were incubated with extracts or reference compounds for 48 h and [$^3$H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of incubation (Benoit-Vical et al., 2007). Parasite growth was estimated by [$^3$H]-hypoxanthine incorporation. The control parasite culture (without drug and with 2% DMSO) was referred to as 100 % growth. The $IC_{50}$ values (50 % inhibitory concentration) were graphically determined as concentrations *versus* percentage of parasite growth.

### 2.4. In vitro anti-Babesia divergens activity

*Babesia divergens* (Rouen 1986 strain) was cultured continuously according to Précigout et al. (Precigout et al., 1991). The parasites were maintained *in vitro* in human red blood cells ($O^{\pm}$; EFS; Toulouse, France), diluted to 6 % haematocrit in RPMI 1640 medium (Biowest, France) supplemented with 30 nM $NaHCO_3$ and complemented with 10 % human $AB^+$ serum (EFS).

The antibabesial activity of extracts was evaluated by the same radioactive micromethod as for *Plasmodium* in 96-well culture plates except for the incubation time, which was reduced to 24 h. The drug-free control parasite cultures were considered as 100 % growth, and an imidocarb sensitivity control was included in every plate. $IC_{50}$ were determined graphically from concentration *versus* percent inhibition curves.

### 2.5. Antileishmanial activity on promastigote stages

The effect of the compounds tested on the growth of *Leishmania infantum* (line MHOM/MA/67/ITMAP-263) promastigotes was assessed by luciferase assay. Briefly, promastigotes in log-phase in RPMI 1640

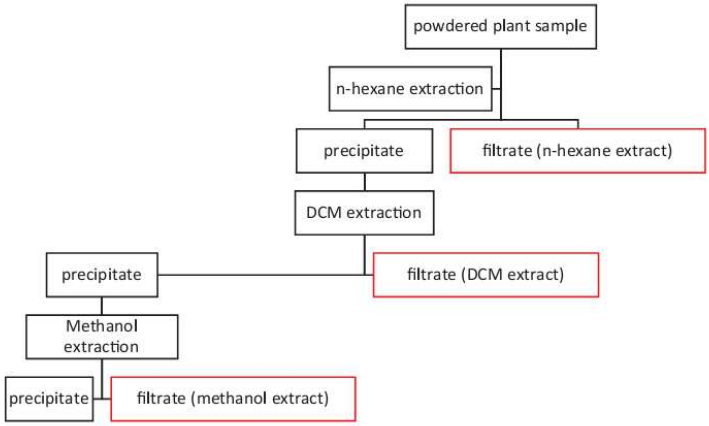



**Fig. 2.** Representative scheme of *P. cauliflora* extraction.
DCM: dichloromethane
Red boxes correspond to extracts used for antiparasitic activity investigations

medium (Biowest, France) supplemented with 10 % fetal calf serum (Hyclone, ThermoFischer, France), 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL geneticin (Sigma, France)) were incubated at an average density of $10^6$ parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO or MeOH (final concentration less than 0.5 % v/v), in duplicate. Appropriate controls treated by DMSO, MeOH and amphotericin B (reference drug purchased from Sigma Aldrich) were added to each set of experiments. After a 72 h incubation period at 24 °C, each plate well was then microscope-examined for any possible precipitate formation. To estimate the luciferase activity of promastigotes, 80 µl of each well were transferred to white 96-well plates, Steady Glow reagent (Promega) was added according to the manufacturer's instructions, and the plates were incubated for 2 min. Luminescence was measured in a Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50 % ($IC_{50}$) was defined as the concentration of drug required to inhibit 50 % of the metabolic activity of *Leishmania infantum* promastigotes compared to the control. $IC_{50}$ were calculated by non-linear regression analysis processed on dose–response curves, using TableCurve 2D V5® software. $IC_{50}$ values are the geometric mean values calculated from three independent experiments.

### 2.6. Cytotoxic evaluation on macrophages and VERO cell lines

The cytotoxicity tests were performed on macrophages and VERO cell lines for compounds showing the best antileishmanial and antiplasmodial activities, respectively. The evaluation of cytotoxicity by MTT assay on the J774A.1 cell line (mouse macrophage cell line, Sigma-Aldrich) was evaluated according to Mosmann (Mosmann, 1983) with slight modifications, and cytotoxicity on VERO cells (monkey epithelial cell line, Sigma-Aldrich) was assessed using [$^3$H]-hypoxanthine incorporation. Briefly, cells ($5.10^4$ cells/mL) in 100 µL of complete medium were seeded into each well of 96-well plates and incubated at 37 °C in a humidified 5 % $CO_2$ with 95 % air atmosphere. The complete medium consisted of RPMI 1640 supplemented with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) for J774A.1 cell line and MEM with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), NEAA 1X for VERO cell line. After 24 h incubation, 100 µL of medium with various product concentrations and appropriate controls (DMSO and doxorubicin) were added and the plates incubated for 72 h at 37 °C, 5 % $CO_2$. For cytotoxic evaluation on macrophages, each plate well was then microscope-examined to detect any precipitate formation before the medium was aspirated from the wells. One hundred µL of MTT solution (0.5 mg/ml in RPMI) was then added to each well and the cells incubated for 2 h at 37 °C. Then the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting formazan crystals. The plates were shaken vigorously for 5 min and absorbance was measured at 570 nm with a microplate spectrophotometer (EON).

For cytotoxic evaluation on VERO cells, the plates were incubated with the test compounds for 48 h and [$^3$H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of the incubation (Benoit-Vical et al., 2007) to estimate VERO cell growth. The control VERO cell culture (drug-free but with 2 % DMSO) was referred to as 100 % of growth. The $IC_{50}$ values (50 % inhibitory concentration) were graphically determined as concentrations *versus* percentage of parasite growth.

## 3. Results and discussion

Antiplasmodial activities of *T. diversifolia, C. barbata, T. crispa, A. flava* and *P. cauliflora* extracts collected from Indonesia are reported in Table 1. Seven out of the 25 crude extracts from these 5 plants showed very interesting activities against *P. falciparum* with $IC_{50}$ values less than the threshold of 5 µg/ml (Willcox et al., 2011). These best antiplasmodial extracts were also tested on the VERO cell line in order to evaluate the specificity of their activity. *T. diversifolia* ethanol and ethyl acetate extracts were active against *P. falciparum* but their cytotoxic values were equivalent (selectivity index less than 1). The ether extract of aerial parts of this plant had already demonstrated *in vitro* antiplasmodial activity ($IC_{50}$: 0.75 µg/ml on the chloroquine-sensitive strain FCA). Those findings led to the extraction and identification of the active compound: taginin C, a sesquiterpene lactone with an $IC_{50}$ of 0.33 µg/ml against FCA strain (Goffin et al., 2002). The ethanol extract of *T. diversifolia* was active at 200 mg/kg *in vivo* in a mouse malaria model (Elufioye and Agbedahunsi, 2004). However, because of the low selectivity index of *T. diversifolia*, this plant presents a limited interest for further pharmacological investigations. *A. flava* ethanol, N-hexane and ethyl acetate (Fig. 3) extracts demonstrated a good antiplasmodial activity ($IC_{50}$ around 3 µg/ml) and interestingly a high selectivity regarding their activities against the VERO cell line (> 50 µg/ml). *A. flava* possesses alkaloid compounds having antioxidant and cytotoxic activities (Keawpradub et al., 2015). Other findings revealed that *A. flava* extracts have $IC_{50}$ ranging from 0.4 to 8.6 µg/mL against *P. falciparum* (Nguyen-Pouplin et al., 2007) and one of its active compounds, berberine, inhibits telomerase activity in *P. falciparum* (Sriwilaijareon et al., 2002). *P. cauliflora* showed the best antiplasmodial $IC_{50}$ values with dichloromethane and methanol extractions particularly of the radix part (3.3 µg/ml with both solvents) and the weakest cytotoxicity for the radix dichloromethane extract (> 50 µg/ml). Bisbenzylisoquinoline alkaloids of *P. cauliflora, i.e.* obaberine and pycnarrhine (Siwon et al., 1981a) have been reported to be responsible for various activities involving antioxidant and cytotoxic properties (Masriani and Adnyana, 2011) but no studies to date have revealed its antiplasmodial activity. This novel plant thus appears to be particularly promising and deserves further study.

Surprisingly and despite their traditional use for malaria treatment, no extracts from *C. barbata* (stem and leaf) or *T. crispa* (stem) showed antiplasmodial activity. Previous studies have reported alkaloid extraction from the root of *C. barbata* with both cytotoxic and antimalarial activities. Five bisbenzylisoquinoline alkaloids were isolated as active principals: tetrandrine, limacine, thalrugosine, homoaromaline, cycleapeltine (Lin et al., 1993). Some of these bisbenzylisoquinoline alkaloids were reported elsewhere for their high antiplasmodial activity (Fang and Jiang, 1986; Marshall et al., 1994). However, *T. crispa* extracts showed *in vivo* antiplasmodial activity of stems against *P. berghei* (Marthianti, 2006) and also *in vitro* but the $IC_{50}$ value of 0.8 mg/mL reported for methanol stem extract (Najib Nik et al., 1999) is far too high to have a pharmacological interest and is thus in accordance with our results.

Because *Babesia* is another protozoan apicomplexan parasite that infects erythrocytes and also causes haemolytic disease, it was interesting also to determine the activity of these plant extracts against *Babesia in vitro*. The 8 extracts showing the best antiplasmodial activity were tested on *Babesia divergens* culture (Table 2). With the exception of *T. diversifolia* ethanol extract, for which the standard deviation of the results was too large to conclude, all the extracts tested showed $IC_{50}$ values ranging from 1.2 to 14.1 µg/ml. These data are particularly interesting because they concern crude extracts and are not so far from the $IC_{50}$ of the control drug imidocarb (0.5 µg/ml) and better than those already reported with other Indonesian medicinal plant extracts (Murnigsih et al., 2005; Murnigsih et al., 2005; Subeki et al., 2007). Our results suggested a strong correlation between antiplasmodial and antibabesial activities and so the potential use of antibabesial compounds from the antiplasmodial drugs portfolio. Moreover, because *Babesia* is an erythrocyte parasite sharing numerous biochemical pathways with *Plasmodium* with the notable exception of the haemoglobin degradation pathways, we could hypothesise that these extracts act on parasitic pathways that do not involve hemozoin polymerisation.

The same 25 extracts tested against *P. falciparum* were also

**Table 1**
Antiplasmodial activity against *P. falciparum*, chloroquine-resistant strain (FCM29-Cameroon strain), and cytotoxicity on VERO cell line

| Extract | $IC_{50}$, µg/mL; Mean[a] ± SD | | | | | | |
|---|---|---|---|---|---|---|---|
| | Antiplasmodial activity/Cytoxicity | | | | | | |
| | (Selectivity Index)[b] | | | | | | |
| | *T. diversifolia* (leaf) | *C. barbata* (stem) | *C. barbata* (leaf) | *T. crispa* (stem) | *A. flava* (stem) | *P. cauliflora* (stem) | *P. cauliflora* (radix) |
| EtOH | 3.9 ± 2/3.5 (0.9) | > 50/ND[c] | > 50/ND | 50/ND | 2.8 ± 0.9/ > 50 (> 18) | –[e] | – |
| *n*-hexane | 16.7 ± 6/ND | 13.7 ± 7/ND | 41 ± 8/ND | 18.7 ± 5/ND | 2.8 ± 0.8/ > 50 (> 18) | > 50/ND | > 50/ND |
| Ethyl acetate | 3.3 ± 0.5/3.1 ± 1.7 (0.9) | 27 ± 5/ND | 33 ± 13/ND | > 50/ND | 3 ± 0.7/ > 50 (> 18) | – | – |
| Residue | > 50/ND | > 50/ND | > 50/ND | > 50/ND | – | – | – |
| Dichloromethane | – | – | – | – | – | 10.5 ± 8/17.5 ± 2 (1.7) | 3.3 ± 0.1/ > 50 (> 15) |
| Methanol | – | – | – | – | – | > 50/ND | 3.3 ± 0.3/6.5 (2) |
| Chloroquine (drug control) | 0.28 ± 0.03[d]/69.3 ± 21 (245) | | | | | | |

[a] Mean of at least 3 independent experiments.
[b] Selectivity Index: Cytotoxicity/activity ratio.
[c] Not done.
[d] Corresponding to the $IC_{50}$ value of 0.51 ± 0.04 µM.
[e] No corresponding extract.

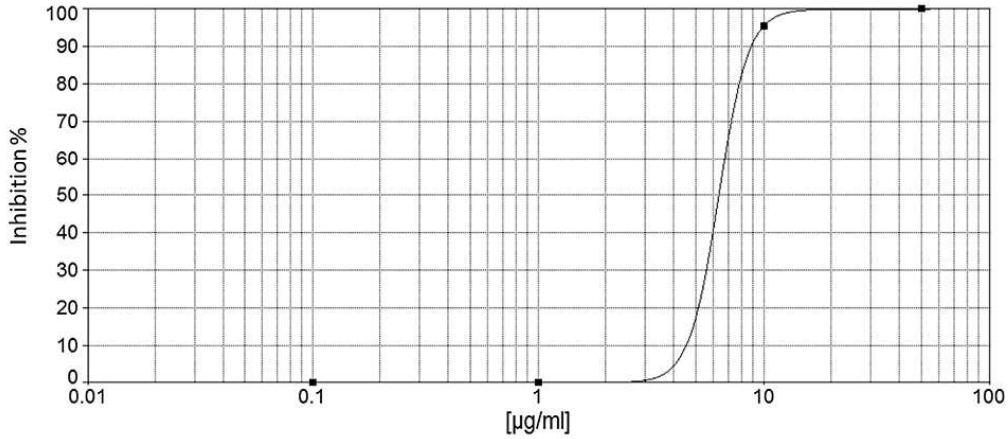



**Fig. 3.** Representative curve of percentage inhibition of *Plasmodium falciparum* growth *in vitro* by *A. flava* ethyl acetate extract.

**Table 2**
Anti-*Babesia* activity against *B. divergens*.

| Extract | $IC_{50}$, µg/mL; Mean[a] ± SD |
|---|---|
| *T. diversifolia* ethanol extract | 36.2 ± 23 |
| *T. diversifolia* ethyl acetate extract | 1.7 ± 0.7 |
| *A. flava* ethanol extract | 6.4 ± 2.8 |
| *A. flava n*-hexane extract | 4.8 ± 2.9 |
| *A. flava* ethyl acetate extract | 6.3 ± 3.2 |
| *P. cauliflora* dichloromethane extract (stem) | 1.5 ± 1 |
| *P. cauliflora* dichloromethane extract (radix part) | 14.1 ± 9 |
| *P. cauliflora* methanol extract (radix part) | 1.2 ± 0.7 |
| Imidocarb (drug control) | 0.5 ± 0.5[b] |

[a] Mean of at least 3 independent experiments.
[b] Corresponding to the $IC_{50}$ value of 1.4 µM.

evaluated against *L. infantum* and in parallel on its macrophage host cell (Table 3). Only 3 extracts out of 25 showed $IC_{50}$ values of less than 50 µg/ml. Dichloromethane (Fig. 4) and methanol fractions of *P. cauliflora* radix showed a good activity on *L. infantum* with $IC_{50}$ values of 1.7 and 4.4 µg/ml, respectively. However the selectivity indexes

**Table 3**
Antileishmanial activity against *L. infantum* promastigote and cytotoxicity on murine J774A.1 macrophage line.

| Extract[b] | $IC_{50}$, µg/mL; Mean[a] ± SD Antileishmanial activity/Cytoxicity (Selectivity Index) |
|---|---|
| *T. diversifolia* ethyl acetate extract | 21.9 ± 1.2/3.3 ± 1.4 (0.03) |
| *P. cauliflora* dichloromethane extract (radix part) | 1.7 ± 0.5/5.8 ± 0.9 (3.4) |
| *P. cauliflora* methanol extract (radix part) | 4.4 ± 0.9/12.4 ± 1.5 (2.8) |
| Amphotericin B (drug control) | 0.06 ± 0.01/3.3 ± 1.4 (55) |

[a] Mean of at least 3 independent experiments.
[b] All extracts tested against *P. falciparum* (see Table 1) were also evaluated against *L. infantum* promastigote. With the exception of those reported here in Table 3, all the other extracts showed $IC_{50}$ greater than 50 µg/ml against *Leishmania* (data not shown) and were not tested for their cytotoxicity.

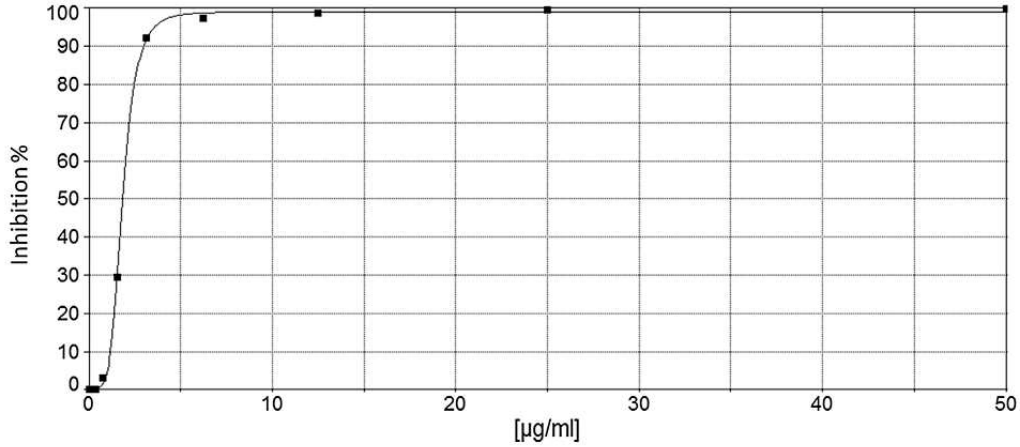



**Fig. 4.** Representative curve of percentage inhibition of *Leishmania infantum* promastigote growth *in vitro* by *P. cauliflora* dichloromethane extract (radix part).

obtained on macrophages are relatively low (around 3). The analysis of these data shows that both best extracts (*P. cauliflora*) on *Leishmania* were also active, at similar concentrations, on *Plasmodium*. It is particularly interesting since the anti-parasite activity of this plant (whether on *Plasmodium*, *Babesia*, or *Leishmania*) has thus far never been reported. Moreover Malaria and Leishmaniasis are co-endemic in Indonesia and the same plant extracts locally present could fight both pathogens.

## 4. Conclusions

Based on an ethnopharmacology approach, 7 plant extracts out of 25 tested displayed good antimalarial activities with $IC_{50} < 5$ µg/ml. Activities observed with ethanolic crude extracts, the extraction solvent studied here that was closest to the aqueous traditional extraction, could justify the traditional use of both plants *T. diversifolia* and *A. flava* for malaria treatment. However regarding their cytotoxicity against mammalian cells, *A. flava* appeared to be the most promising antiplasmodial plant of this study with the highest antiplasmodial activity ($IC_{50}$ values less than 3 µg/ml) and the lowest cytotoxicity. Moreover, for all extracts showing antiplasmodial activity a positive correlation was found with antibabesial activity, suggesting that antiplasmodial extracts can be a potential source of antibabesial compounds. In contrast, *A. flava* had no activity on *L. infantum*. Furthermore, only *P. cauliflora* radix, through its dichloromethane and methanol fractions also demonstrated a good activity against *L. infantum*, with $IC_{50}$ values equivalent for the 3 pathogens; their selectivity index, especially on VERO cells, suggests a specific parasiticidal action more than a global cytotoxic effect.

These preliminary results are sufficiently interesting to pursue phytochemical investigations in order to identify the chemical series responsible for these activities and obtain standardized preparations. This is an absolute necessity before considering any therapeutic applications to malaria, leishmaniasis and/or babesiosis for either herbal preparations or pure molecules.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

This work was successfully carried out thanks to a multidisciplinary and multinational collaboration involving Indonesian and French teams with the financial support of Campus France PHC-NUSANTARA (Grant 29653YF). A.P. Ramadani obtained a *Beasiswa Unggulan* grant, Bureau of Planning and International Coorperation, the Ministry of Education and Culture of the Republic of Indonesia and is completing her PhD under joint supervision of University Paul Sabatier, Toulouse, France and UGM, Yogyakarta, Indonesia.

The authors also thank Mariette Purcell for editing the English.

## References

Benoit-Vical, F., Valentin, A., Da, B., Dakuyo, Z., Descamps, L., Mallie, M., 2003. N'Dribala (*Cochlospermum planchonii*) *versus* chloroquine for treatment of uncomplicated *Plasmodium falciparum* malaria. J. Ethnopharmacol. 89, 111–114.

Benoit-Vical, F., Lelievre, J., Berry, A., Deymier, C., Dechy-Cabaret, O., Cazelles, J., Loup, C., Robert, A., Magnaval, J.F., Meunier, B., 2007. Trioxaquines are new antimalarial agents active on all erythrocytic forms, including gametocytes. Antimicrob. Agents Chemother. 51, 1463–1472.

Benoit-Vical, F., Soh, P.N., Salery, M., Harguem, L., Poupat, C., Nongonierma, R., 2008. Evaluation of Senegalese plants used in malaria treatment: focus on *Chrozophora senegalensis*. J. Ethnopharmacol. 116, 43–48.

Benoit-Vical, F., 2005. Ethnomedicine in malaria treatment. IDrugs 8, 45–52.

Ceci, L., Iarussi, F., Greco, B., Lacinio, R., Fornelli, S., Carelli, G., 2014. Retrospective study of hemoparasites in cattle in southern Italy by reverse line blot hybridization. J. Vet. Med. Sci. 76, 869–875.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D., 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob. Agents Chemother. 16, 710–718.

Elufioye, T.O., Agbedahunsi, J.M., 2004. Antimalarial activities of *Tithonia diversifolia* (Asteraceae) and *Crossopteryx febrifuga* (Rubiaceae) on mice in vivo. J. Ethnopharmacol. 93, 167–171.

Fang, D.C., Jiang, M.X., 1986. Studies on tetrandrine calcium antagonistic action. Chin. Med. J. 99, 638–644.

Goffin, E., Ziemons, E., De Mol, P., de Madureira Mdo, C., Martins, A.P., da Cunha, A.P., Philippe, G., Tits, M., Angenot, L., Frederich, M., 2002. In vitro antiplasmodial activity of *Tithonia diversifolia* and identification of its main active constituent: tagitinin C. Planta Med. 68, 543–545.

Keawpradub, N., Dej-adisai, S., Yuenyongsawad, S., 2015. Antioxidant and cytotoxic activities of Thai medicinal plants named Khaminkhruea: *Arcangelisia flava, Coscinium blumeanum* and *Fibraurea tinctoria*. J. Sci. Technol. 27, 13.

L'Hostis, M., Chauvin, A., Valentin, A., Marchand, A., Gorenflot, A., 1995. Large scale survey of bovine babesiosis due to *Babesia divergens* in France. Vet. Rec. 136, 36–38.

Lin, L.Z., Shieh, H.L., Angerhofer, C.K., Pezzuto, J.M., Cordell, G.A., 1993. Cytotoxicc and antimalarial bisbenzylisoquinoline alkaloids from *Cyclea barbata*. J. Nat. Prod. 56, 22–29.

Lovin, E.R., Arwati, H., Ramadhani, R.B., 2012. In vitro intraerythrocytic antimalarial activity of akar kuning (*Arcangelisia flava* (L.) Merr.) stem aqueous extract in *Plasmodium falciparum*. Folia Medica Indonesiana 48, 90–95.

Mandia, E.H., 1999. *Arcangelisia flava* (L.) Merr. In: de Padua, L.S., Bunyapraphatsara, N.B., Lemmens, R.H.M.J. (Eds.), Plant Resources of South-East Asia: Medicinal and Poisonous Plants. Backhuys Publisher, Leiden, pp. 129–132.

Manilal, K.S., Sabu, T., 1985. *Cyclea barbata* miers (menispermaceae): a new record of a medicinal plant from South India. Anc. Sci. Life 4, 229–231.

Marshall, S.J., Russell, P.F., Wright, C.W., Anderson, M.M., Phillipson, J.D., Kirby, G.C., Warhust, D.C., Schiff, P.L., 1994. In vitro antiplasmodial, antiamoebic, and cytotoxic activities of a series of bisbenzylisoquinoline alkaloids. Antimicrob. Agents Chemother. 38, 96–103.

Marthianti, A., 2006. The Effects in the Treatment Using Tinospora Crispa's Stem Extract

Compare to Kloroquin on the Amounts of Erythrocyte in the Swiss Mice Which Was Infected with *Plasmodium Berghebi*. Thesis Faculty of medicine Diponegoro University, Indonesia.

Masriani, E., Adnyana, I.K., 2011. Aktivitas antioksidan ekstrak etanol daun sengkubak (*Pycnarrhena cauliflora* (Miers.) Diels) asal Kalimantan Barat.

Melhorn, H., Schein, E., 1984. The piroplasms: life cycle and sexual stages. Adv. Parasitol. 23, 37–103.

Miura, T., Nosaka, K., Ishii, H., Ishida, T., 2005. Antidiabetic effect of Nitobegiku, the herb *Tithonia diversifolia*, in KK-Ay diabetic mice. Biol. Pharm. Bull. 28, 2152–2154.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Murnigsih, T., Subeki Matsuura, H., Takahashi, K., Yamasaki, M., Yamato, O., Maede, Y., Katakura, K., Suzuki, M., Kobayashi, S., Chairul Yoshihara, T., 2005. Evaluation of the inhibitory activities of the extracts of Indonesian traditional medicinal plants against *Plasmodium falciparum* and *Babesia gibsoni*. J. Vet. Med. Sci. 67, 829–831.

Najib Nik, A.R.N., Furuta, T., Kojima, S., Takane, K., Ali Mohd, M., 1999. Antimalarial activity of extracts of Malaysian medicinal plants. J. Ethnopharmacol. 64, 249–254.

Nguyen-Pouplin, J., Tran, H., Phan, T.A., Dolecek, C., Farrar, J., Tran, T.H., Caron, P., Bodo, B., Grellier, P., 2007. Antimalarial and cytotoxic activities of ethnopharmacologically selected medicinal plants from South Vietnam. J. Ethnopharmacol. 109, 417–427.

Njoroge, G.N., Bussmann, R.W., 2006. Diversity and utilization of antimalarial ethnophytotherapeutic remedies among the Kikuyus (Central Kenya). J. Ethnobiol. Ethnomed. 2, 8.

Noor, H., Ashcroft, S.J., 1998. Pharmacological characterisation of the anti-hyperglycaemic properties of *Tinospora crispa* extract. J. Ethnopharmacol. 62, 7–13.

Oyewole, I.O., Ibidapo, C.A., Moronkola, D.O., Oduola, A.O., Adeoye, G.O., Anyasor, G.N., Obansa, J.A., 2008. Anti-malarial and repellent activities of *Tithonia diversifolia* (Hemsl.) leaf extracts. J. Med. Plants Res. 2, 171–175.

Pathak, A.K., Jain, D.C., Sharma, R.P., 1995. Chemistry and biological activities of the genera *Tinospora*. Int. J. Pharmacognosy 33, 277–287.

Precigout, E., Gorenflot, A., Valentin, A., Bissuel, G., Carcy, B., Brasseur, P., Moreau, Y., Schrevel, J., 1991. Analysis of immune responses of different hosts to *Babesia divergens* isolates from different geographic areas and capacity of culture-derived exoantigens to induce efficient cross-protection. Infect. Immun. 59, 2799–2805.

Pushpangadan, P., Atal, C.K., 1984. Ethno-medico-botanical investigations in Kerala I: Some primitive tribals of western ghats and their herbal medicine. J. Ethnopharmacol. 11, 59–77.

Rungruang, T., Boonmars, T., 2009. In vivo antiparasitic activity of the Thai traditional medicine plant – *Tinospora crispa*– against *Plasmodium yoelii*. Southeast Asian. J. Trop. Med. Public Health 40, 898–900.

Saxena, S., Pant, N., Jain, D.C., Bhakuni, R.S., 2003. Antimalarial agents from plant sources. Curr. Sci. 85, 1314–1329.

Siwon, J., Verpoorte, R., Svendsen, A.B., 1981a. Studies on Indonesian medicinal plants VI further alkaloids from *Fibraurea chloroleuca*. Planta Med. 41, 65–68.

Siwon, J., Verpoorte, R., Vanbeek, T., Meerburg, H., Svendsen, A.B., 1981b. Studies on Indonesian medicinal-plants. 4. Alkaloids from *Pycnarrhena-Longifolia*. Phytochemistry 20, 323–325.

Sriwilaijareon, N., Petmitr, S., Mutirangura, A., Ponglikitmongkol, M., Wilairat, P., 2002. Stage specificity of *Plasmodium falciparum* telomerase and its inhibition by berberine. Parasitol. Int. 51, 99–103.

Straimer, J., Gnadig, N.F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A.P., Dacheux, M., Khim, N., Zhang, L., Lam, S., Gregory, P.D., Urnov, F.D., Mercereau-Puijalon, O., Benoit-Vical, F., Fairhurst, R.M., Menard, D., Fidock, D.A., 2015. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. Science 347, 428–431.

Subeki Matsuura, H., Takahashi, K., Yamasaki, M., Yamato, O., Maede, Y., Katakura, K., Suzuki, M., Trimurningsih Chairul Yoshihara, T., 2005. Antibabesial activity of protoberberine alkaloids and 20-hydroxyecdysone from *Arcangelisia flava* against Babesia gibsoni in culture. J. Vet. Med. Sci. 67, 223–227.

Subeki Matsuura, H., Takahashi, K., Nabeta, K., Yamasaki, M., Maede, Y., Katakura, K., 2007. Screening of Indonesian medicinal plant extracts for antibabesial activity and isolation of new quassinoids from *Brucea javanica*. J. Nat. Prod. 70, 1654–1657.

The Southwest School of Botanical Medicine, 1918. The Dispensatory of the United States of America.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Vennerstrom, J.L., Klayman, D.L., 1988. Protoberberine alkaloids as antimalarials. J. Med. Chem. 31, 1084–1087.

Verpoorte, R., Vanrijzen, A.H.M., Siwon, J., Baerheimsvendsen, A., 1978. Tertiary alkaloids of Pycnarrhena Novoguineensis. 1. Studies on indonesian medicinal plants. Planta Med. 34, 274–279.

Verpoorte, R., Siwon, J., Vanessen, G.F.A., Tieken, M., Svendsen, A.B., 1982a. Studies on Indonesian medicinal-plants. 7. Alkaloids of *Arcangelisia-Flava*. J. Nat. Prod. 45, 582–584.

Verpoorte, R., Vanbeek, T.A., Siwon, H., Svendsen, A.B., 1982. Studies on Indonesian Medicinal-Plants.8. Screening of Some Indonesian Menispermaceae for Alkaloids – the Isolation of Magnoflorine from *Pycnarrhena Novoguinensis* and *Cyclea Barbata*. Pharmaceutisch Weekblad-Scientific Edition 4, 87–88.

Vial, H.J., Gorenflot, A., 2006. Chemotherapy against babesiosis. Vet. Parasitol. 138, 147–160.

WHO, 2014. Leishmaniasis.

WHO, 2014. Status report on artemisinin resistance. WHO/HTM/GMP/2014.9 Global Malaria Programme.

WHO, 2014. World Malaria Report 2014. 142 p.

Willcox, M., Benoit-Vical, F., Fowler, D., Bourdy, G., Burford, G., Giani, S., Graziose, R., Houghton, P., Randrianarivelojosia, M., Rasoanaivo, P., 2011. Do ethnobotanical and laboratory data predict clinical safety and efficacy of anti-malarial plants? Malar. J. 10 (Suppl 1), S7.

Witkowski, B., Berry, A., Benoit-Vical, F., 2009. Resistance to antimalarial compounds: methods and applications. Drug Resist. Updat. 12 (1–2), 42–50.

Wongbutdee, J., Sujarit, K., Tuchinda, P., Thinapong, P., Piyachaturawat, P., 2003. Hepatoprotective effect of *Arcangelisia flava* Merr extracts in mice. Thai. J. Physiol. Sci. 26, 56.

Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R., 2002. Epidemiology of drug-resistant malaria. Lancet Infect. Dis. 2, 209–218.

Zintl, A., McGrath, G., O'Grady, L., Fanning, J., Downing, K., Roche, D., Casey, M., Gray, J.S., 2014. Changing incidence of bovine babesiosis in Ireland. Ir. Vet. J. 67, 19.

## B. Antiplasmodial Activity of Gold(I) Complexes

1. Results obtained

Synthetic molecules are part of another approach to discover new antimalarial drugs. Organometallic compounds containing metal complex that acts as pro-drugs and can have an enhanced efficacy (such as ferrocene with CQ leading not only to ferroquine but also gold and ruthenium complexes). NHCs, as the most important ligands in organometallic chemistry, are easy to prepare and able to stabilize the complexes. Biomedical applications of gold complexes based on NHCs are beginning to unfold. In particular, these ligands form strong Au-carbene bonds, giving stable $Au^{I}$-NHCs complexes that are insensitive to biologically important thiol groups. $Au^{I}$-NHC have shown potential medical applications [183], especially as anticancer [182, 184-187] and antimicrobial agents [189]. It has to be mentioned that gold-based compounds, including some gold(I)-NHCs, show anti-mitochondrial activity, a promising mode of action to fight cancer. Their antitumor activity may stem from the lipophilic and cationic properties, allowing their accumulation in mitochondria of tumor cells with great specificity [183]. It has also been demonstrated that the combination of metal complexes with NHCs can exhibit interesting biological activities against such parasitic infections as malaria and leishmaniasis [190, 204-206].

Our previous study proved that mononuclear cationic gold(I) complexes containing aromatic amino-functionalized NHC have *in vitro* antiplasmodial activity against FCM29-Cameroon [190]. This finding is exploited in this present study by further studies and fine tuning of the main structure of these organometallic molecules in relation to their antiplasmodial activity or against other parasites as well as studying the activity of new complexes.

To achieve the objective of investigating the influence of the different structure modulations on biological activities, synthesized proligand imidazolum salts (compounds **1-**

**11**) and mononuclear gold(I) complexes (compounds **12**-**25**) were evaluated for its activity against CQ-resistant *P. falciparum* strains (FcM29-Cameroon and FcB1-Colombia) and put in comparison with CQ and artemisinin. These synthesized compounds (Fig. 12-14) were a series of N-, O- or S-functionalized proligands and their corresponding mononuclear cationic, anionic or neutral gold(I) bisNHC complexes. The most active compound would be investigated on its cytotoxic activity on Vero or McF7 cell lines.

Compound **1** (2-chloroquinoline) is an imidazolum precursor that is functionalized by nitrogen containing heterocycles quinoline. An aryl directly attached to its azolium ring generates compound **2** and **3**. As another imidazolum precursor, compound **4** is containing bipyridine. Meanwhile, compound **5 – 8** are the amine functionalized imidazolium salt combine with alkyl- (metyl for compound **5** and n-butyl for **6**) and arylimidazoles (benzyl for compound **7** and mesityl for **8**).

Using those imidazolium precursors, gold(I) complexes are developed. Compound **1** generates compound **12**, **2** to **14**, **3** to **15**, **4** to **16**, **5** to **18**, **6** to **19**, **7** to **20**, and **8** to **21**. Meanwhile compound **13** and **17** are neutral form of the complexes. In addition, imidazolium salt compound **9, 10, 11** are precursor for compound **22** and **23**; **24**; and **25** respectively.

Assessed against *P. falciparum* strains FcM29-Cameroon and FcB1-Columbia, different compounds (**4**, **5**, **6**, **18**, **19**) indicated various results allowing relationship structure activity studies. As cationic molecule, the imidazolium salts (proligand **1** and **4**-**11**) were inactive while proligands **2** and **3** showed $IC_{50}$ value of 1.4 and 1.5 µM respectively. Tested gold(I) complex compounds (**12**, **15**-**16** and **19**-**22**) expressed improved activities compared to imidazolium salt demonstrating that gold cation plays an important role in antiplasmodial activity. A prior study suggested that one of the possible mechanisms of action of gold compounds is based on the reversible inhibition of the parasite enzyme falcipain 2 (Fp2)

involved in hemoglobin digestion. As a cysteine protease of *P. falciparum*, Fp2 will provide a significant quantity of peptides and amino acids from human hemoglobin degradation for parasite growth. Moreover, it is also characterized as a validated target for antimalarial drug discovery [191].

It has been demonstrated that cationic compounds can enhance antiplasmodial activities by positively facilitating the accumulation of molecules inside the parasite, the global charge thus become the key factor. It has been shown that the cationic Au(I) bis (NHC) species had the best results against *P. falciparum* with sub-micro molar $IC_{50s}$ for some of them were 2.1 µM (**19**), 0.48 µM (**20**), 0.21 µM (**21**), and 0.32 µM (**22**). On the contrary, moderate activities were obtained with neutral complexes (compounds **13**, **17** and **23** with $IC_{50}$ between 4.1 and 6.6 µM) as well as anionic compounds **24** and **25** ($IC_{50}$ 5.6 µM and 3.7 µM, respectively).

The NHC carbenic ligand not only acted as a transporter of metal center to its target but also affected antiplasmodial efficacy proven by the gold(I) series **18**-**21** reducing $IC_{50}$ values from methyl- (**18**: 22 µM) to mesityl – substitutes (**21**: 0.21 µM). The complex **21,** the most active compound against *P. falciparum,* had lipophilic properties allowing to penetrate into the parasite through its different membranes.

Another compound with nitrogen-functionalized NHC revealed that a quinoline moiety in the complex had a lower antiplasmodial activity (compound **13**, $IC_{50}$: 5.2µM) than compounds **12** and **14** ($IC_{50}$ 1.1µM) which had methyl and mesityl as non-functionalized substituents. Compound **15** ($IC_{50}$: 0.47µM) with a thioether group demonstrated an increased antiplasmodial activity, and a methylthiophenyl group was responsible for the antiplasmodial potency of compound **22** ($IC_{50}$: 0.32µM).

The most active compounds (**15**, **16**, **20**, **21** and **22**) were also tested for their specificity against mammalian cell lines. The selectivity index was ranked from 0.9 to 18 with the highest

selectivity index for compound **21**. These findings supported previous studies of cancer cell lines ($IC_{50}$ < 1µM) and non-tumor cell lines ($IC_{50}$ > 5µM) [204]. It means that complex **21** is particularly active against *P. falciparum* or several cancer cell lines but having limited effects on normal human cells.

It is noted that cationic lipophilic gold(I)-NHC complexes are the most active compounds against *P. falciparum* compared to both neutral and anionic compounds. The lipophilicity property and presence of nitrogen or sulfur atoms in the ligands were likely to be responsible for the antiplasmodial activity as detailed in the following published article.

2. Corresponding publication

Bioorganic & Medicinal Chemistry 24 (2016) 3075–3082

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Antiplasmodial activities of gold(I) complexes involving functionalized *N*-heterocyclic carbenes

Catherine Hemmert [a,b,*], Arba Pramundita Ramadani [a,b,c], Luca Boselli [a,b], Álvaro Fernández Álvarez [a,b], Lucie Paloque [a,b], Jean-Michel Augereau [a,b], Heinz Gornitzka [a,b,*], Françoise Benoit-Vical [a,b,*]

[a] CNRS, LCC (Laboratoire de Chimie de Coordination), 205 route de Narbonne, BP 44099, F-31077 Toulouse Cedex 4, France
[b] Université de Toulouse, UPS, INPT, F-31077 Toulouse Cedex 4, France
[c] Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

ARTICLE INFO

*Article history:*
Received 21 April 2016
Accepted 14 May 2016
Available online 17 May 2016

*Keywords:*
*N*-Heterocyclic carbene
Gold
Organometallic complexes
Antiplasmodial drugs

ABSTRACT

A series of twenty five molecules, including imidazolium salts functionalized by N-, O- or S-containing groups and their corresponding cationic, neutral or anionic gold(I) complexes were evaluated on *Plasmodium falciparum* in vitro and then on Vero cells to determine their selectivity. Among them, eight new compounds were synthesized and fully characterized by spectroscopic methods. The X-ray structures of three gold(I) complexes are presented. Except one complex (**18**), all the cationic gold(I) complexes show potent antiplasmodial activity with $IC_{50}$ in the micro- and submicromolar range, correlated with their lipophilicity. Structure–activity relationships enable to evidence a lead-complex (**21**) displaying a good activity ($IC_{50}$ = 210 nM) close to the value obtained with chloroquine ($IC_{50}$ = 514 nM) and a weak cytotoxicity.



## 1. Introduction

According to the latest estimates, malaria led to about 500,000 deaths in 2015 with 70% of all deaths for children aged under 5 years.[1] Thanks to global fight against vectors and the large use of artemisinin-based combination therapies (ACT), estimated malaria mortality rates decreased by 60% worldwide between 2000 and 2015.[1] Unfortunately, ACT resistance is now reported in many South-east Asia countries whereas there are no other antiplasmodial drugs to replace them.[1] New drugs with alternative chemical structures are urgently needed.

The peculiar biological properties of some organometallic compounds have helped in the development of new drugs based on metal complexes for major medical human problems including mainly cancer along with bacterial, viral and parasitic infections.[2] Generally, metal complexes are subject to modifications (ligand substitution and/or redox reactions) during uptake and transport inside the human body; this will influence the reactivity of the metal, but also greatly affect the absorption and delivery of the complex, and sometimes even the specificity of target recognition so they are therefore almost always 'pro-dugs'. Metals may also be used to enhance the efficacy of known organic drugs and the metal-drug synergism results in the enhancement of the activity of the parental organic drug due to binding to the metal ion. More specifically, numerous metal-containing compounds have been evaluated as antimalarial agents and a recent review overviewed the advancements in this field during the last three decades.[3] Among metal coordination complexes in which the metal center is directly coordinated to a known organic drug, gold and ruthenium complexes containing chloroquine (CQ) or analogs were the most active (with $IC_{50}$ in the nM range), with improved efficacy, when compared to the drug, against CQ-sensitive and CQ-resistant strains, in vitro and in vivo.[4] In particular, the principal mechanism proposed for the antimalarial action of $[Au(CQ)(PPh_3)]PF_6$ against resistant strains of *Plasmodium falciparum* is the interaction with heme and the inhibition of β-hematin formation. Both the enhanced activity and the ability of this compound to lower CQ-resistance are related to the high lipophilicity of the metal complex and the important structural modification of the CQ structure imposed by the presence of the metal-containing fragment.[4a] A great success was obtained by C. Biot and coll. with impressive biological results in the introduction of a ferrocenyl moiety into the lateral side chain of CQ, leading to the bioorganometallic drug Ferroquine (FQ) which is more active than CQ, and equally active against CQ-susceptible and CQ-resistant clones.[5] The potent activity of FQ and the absence of cross-resistance with other

* Corresponding authors.
*E-mail addresses:* Catherine.Hemmert@lcc-toulouse.fr (C. Hemmert), Heinz.Gornitzka@lcc-toulouse.fr (H. Gornitzka), Francoise.Vical@inserm.fr (F. Benoit-Vical).

http://dx.doi.org/10.1016/j.bmc.2016.05.023
0968-0896/

antimalarials were reported both in vitro on a large number of clones and field isolates of *P. falciparum*, and in vivo on rodent models that demonstrated the high bioavailability of the product.[5d] FQ has been developed by Sanofi and is the only metallodrug that has entered clinical trials (Phase IIb) against malaria, currently evaluated in combination with Artefenomel (OZ439).[5f] FQ has a multi-mode of action: a capacity to target lipids, an ability to inhibit the formation of hemozoin, and to generate reactive oxygen species.[5e,6]

NHCs represent one of the most important classes of ligands in organometallic chemistry. The easy preparation of NHC-precursors has allowed an almost infinite access to new organometallic complexes, in which the nitrogen atoms of the azoliums rings can be functionalized by organic groups, organometallic moieties and biologic entities. NHCs are extremely good σ-donors, making strong metal–carbene bonds and giving stable complexes in biological medium. Numerous NHC–metal complexes exhibit very interesting biological activities as reflected in several review articles.[7] By varying the properties of the NHCs and the nature of the metals (mainly Cu, Ag, Au, Ni, Pd, Pt and Ru), different important diseases have been targeted, mainly fungi,[7b,i] bacteria,[7b–d,i] and cancer.[7a–f,h–k] We focused our research on heteroditopic *N*-heterocyclic carbene (NHC) gold(I) complexes for their biomedical potential in anticancer and parasitic diseases such as malaria and leishmaniasis.[8] New gold(I) complexes containing two 1-[2-(diethylamino)ethyl]imidazolydene ligands have been synthetized and their anticancer activity reported in five representative human cancer cell lines (prostate, breast, brain, lung and liver).[8a] Very recently, we have demonstrated that mononuclear cationic and especially neutral gold(I) NHC-quinoline proved to be promising metallodrugs with potent and selective action against the pathological relevant form (intramacrophagic amastigote) of *Leishmania*.[8d] Moreover, we have previously synthetized and tested against *Plasmodium falciparum* dinuclear dimeric and mononuclear gold(I) complexes and we have shown that mononuclear cationic gold(I) complexes containing aromatic amino-functionalized NHC exhibit in vitro potent antiplasmodial activity against the chloroquine-resistant strain FcM29-Cameroon with $IC_{50}$ values up to 330 nM.[8c]

The rational design of new gold *N*-functionalized NHCs complexes with specific and/or selective parasite targets appears thus as a very useful avenue in the antiplasmodial drug research.

In this work, we aim to extend our preliminary work concerning gold(I) NHCs complexes against *Plasmodium falciparum* by fine tuning and studying the key building blocks of these organometallic molecules. For this purpose, we report the antiplasmodial activity of our gold(I) complexes already described for other biomedical applications.[8c] In addition, we report the preparation, characterization and antimalarial potential of new mononuclear cationic, anionic and neutral gold(I) NHC or bis(NHC) complexes.

## 2. Results and discussion

### 2.1. Chemistry and structural characterization

The synthesis of proligands **1**–**8** was already described (Scheme 1).[8a,c,d] The imidazolium salts **9** and **11** were simply obtained after a quaternization step of 1-arylimidazole, namely 1-(4-methylthiophenyl)-1*H*-imidazole and 1-(4-hydroxyphenyl) imidazole, respectively, with one equivalent of benzylchloride and $KPF_6$ at 80 °C in acetonitrile (Scheme 2). The zwitterionic proligand **10** was easily synthetized by heating stoichiometric amounts of benzylimidazole and bromoacetic acid at 130 °C, following by deprotonation of the acidic proton by addition of $Na_2CO_3$ under reflux in water (Scheme 2). The most notable features in the $^1H$ and $^{13}C$ NMR spectra of the imidazolium salts are the resonances for imidazolium protons ($H_2$) located at 9.97, 9.21 and 10.16 ppm and the corresponding imidazolium carbons ($C_2$) in the range of 135.6–164.0 ppm, for **9**, **10** and **11** respectively. The high resolution mass spectra (ES) of proligands **9** and **11** exhibit the classical peak corresponding to the cation $[M-PF_6^-]^+$ and that of zwitterion **10** shows the cation $[M+H^+]$.

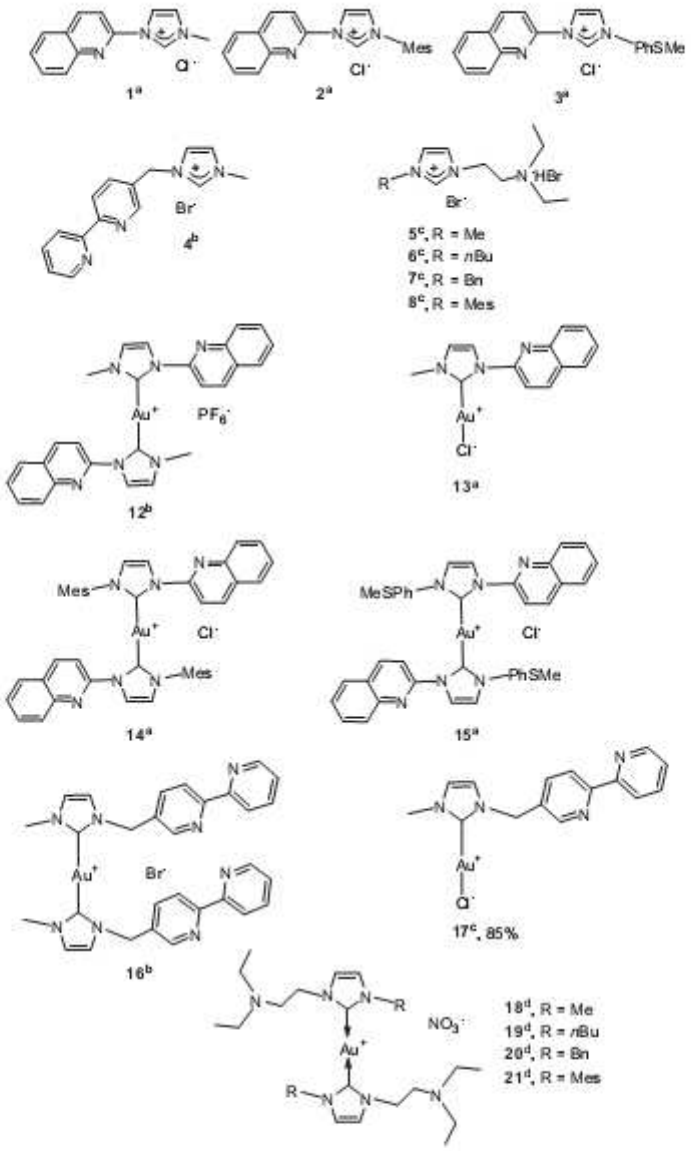
The synthesis of complexes **12**–**16** and **18**–**21** was previously reported in the literature (Scheme 1).[8a,c,d] The mononuclear gold (I) bis(NHC) complexes **22** and **25** (Scheme 2) were prepared according a classical way, from the *N*-functionalized imidazolium salts **9** or **11**, respectively and one half equivalent of $Au(SMe_2)Cl$ with sodium acetate as a mild base in hot *N*,*N*-dimethylformamide (120 °C) and were isolated as white or beige powders with good yields (66–87%). The neutral gold(I) NHC complexes **17** and **23** and the anionic complex gold(I) bis(NHC) **24** (Schemes 1 and 2) were synthetized via the convenient transmetalation route. Firstly, the silver precursor complexes were prepared by deprotonation of the imidazolium salts **4**, **9** or **10**, with one half equivalent of the mild base $Ag_2O$ in a mixture of $CH_2Cl_2$-MeOH at room temperature or in MeOH at 50 °C. For **17**, the silver species $Ag(\mathbf{4})_2Br$[8c] was isolated before the carbene transfer reaction while for compounds **23** and **24**, the transmetalation was carried out in situ, by adding one (for **17** and **23**) or one half equivalent (for **24**) of $Au(SMe_2)Cl$, with respect to the ligand. The three later compounds were obtained as gray solids with high yields (82–85%) and all complexes are stable towards air and moisture. NMR spectroscopy unequivocally demonstrates the formation of the gold(I) complexes; the $^{13}C$ spectra show the resonance for the carbene carbon atoms between 181.6 and 184.5 ppm for the gold(I) bis (NHC) complexes **22**, **24** and **25**, and at 172.0 and 168.9 ppm for the neutral species **17** and **23**, respectively. These values are in the range of reported values for $Au^I$–NHC complexes having C–Au–X (X = halide) or C–Au–C motifs.[9] It has to be mentioned that in the $^1H$ NMR of complex **24**, both protons of the $CH_2COO^-$ become inequivalent. This could be due to the interaction of the two carboxylate with a proton, which may impede the free rotation of the $CH_2$. Moreover, for complex **25** the phenol functions are depronated during the complexation process which is supported by the absence of the OH proton in the $^1H$ NMR spectrum (the OH proton of proligand **11** was located at 10.16 ppm) and by the ES-HRMS spectrum showing the molecular peak $[M+H^+]$ at *m*/*z* 697.1874 with M = $Au^I$bis(NHC-phenolato). The elemental analysis of the gold(I) complexes are in agreement with the proposed structures and the high resolution mass spectra (ES) exhibit the classical peaks corresponding to the cationic fragments $[M+H]^+$ for the neutral and the anionic complexes **17**, **23**–**25** and $[M-PF_6^-]^+$ for the cationic compound **22**.

Crystals of **17** suitable for X-ray diffraction analysis were obtained by slow evaporation of a chloroform solution of **17** (Fig. 1).

Slow evaporation of a MeOH solution of **22** gave crystals suitable for X-ray diffraction analysis (Fig. 2).

Crystals of **23** suitable for X-ray diffraction analysis were obtained by slow evaporation of an acetonitrile solution of **23** (Fig. 3).

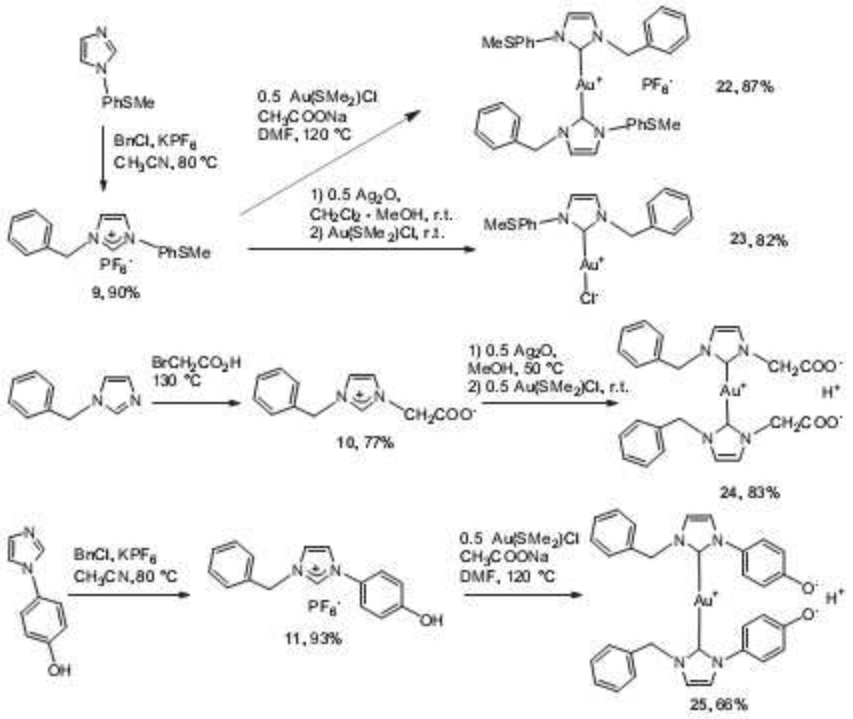
All carbon–gold and chlorine–gold distances, as well as the carbon–gold–carbon and carbon–gold–chlorine angles are in the normal range for such compounds. In **22** the gold cation is located on an inversion center and both NHC ring systems are absolutely coplanar. Moreover, the benzyl substituents are in trans position.




Scheme 1. Proligands and gold(I) complexes used in this study. [a]Ref. 8d, [b]Ref. 8c, [c]this work and [d]Ref. 8a.

### 2.2. Anti-malarial activity and selectivity

Twenty five molecules, involving imidazoliums salts **1–11** and mononuclear gold(I) complexes **12–25** were screened in vitro against *P. falciparum* strains (Table 1) by determining their 50% inhibitory concentrations ($IC_{50}$) and compared to the antimalarial reference-drugs chloroquine and artemisinin. As expected, proligands **1** and **4–11** showed weak or no activity with $IC_{50}$ values ranging from 6.4 to >230 μM, the worst value being obtained for the neutral zwitterionic species **10**, all other imidazoliums salts being cationic. More surprising, the two proligands **2** and **3** containing both a quinoline entity and an aryl directly branched on the azolium ring exhibit interesting antiplasmodial potency with $IC_{50}$ values of 1.4 and 1.5 μM, respectively. As expected, an enhanced activity was observed for the tested gold(I) complexes **12, 15–16** and **19–22** when compared to the corresponding starting proligands, except for the couple **2/14** showing similar activity. This set of results corroborates in most cases the key role of the gold cation in antiprotozoal activity against *P. falciparum*. The three neutral complexes **13, 17** and **23** showed moderate activity with $IC_{50}$ comprised between 4.1 and 6.6 μM and the $IC_{50}$ values of the two anionic compounds **24** and **25** are in the same order of




**Scheme 2.** Synthesis of imidazolium salts **9–11** and gold(I) bis(NHC) complexes **22–25**.

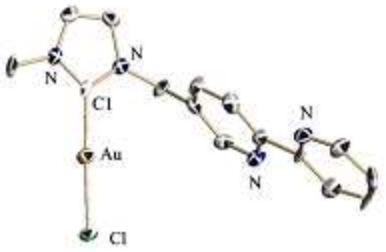



**Figure 1.** Structure of **17** in the solid state, thermal ellipsoids are depicted at a 50% level. Hydrogen atoms have been omitted for clarity. Selected bond lengths [Å] and angles [°]: C1–Au = 1.99(2), Au–Cl = 2.30(1), C1–Au–Cl = 177.7(4).

magnitude, 5.6 μM and 3.7 μM, respectively. With the exception of complex **18**, the best values on *P. falciparum* were obtained for the cationic Au(I)bis(NHC) species with $IC_{50}$ in the micromolar and submicromolar range, with 2.1 μM (**19**), 0.48 μM (**20**), 0.21 μM (**21**), and 0.32 μM (**22**). A first insight can emerge from these results and confirm our preliminary published results (concerning **12** and **16**[9c]): the global charge of the complex constitutes an important factor and cationic compounds are correlated with an increase antiplasmodial activity. The same antiplasmodial properties were reported with albitiazolium, a bis cationic compound witch reached the clinical 2b step for antimalarial drug development. Albitiazolium had shown its entry facilitated by a poly-specific cation transporter present in the parasite membrane.[10] The cationic property could facilitate the accumulation of the molecules inside the parasite where the pH of the cytoplasm is approximately 7.15 but certainly not in the malaria digestive vacuole where pH is about 5.[11] Moreover, even if the carbenic ligand acts mainly as a carrier for the metal center towards its target(s), as it is generally the case for gold complexes with biological potential, the two substituents on the NHC plays a crucial role with regard to the antiparasitic efficiency. This is well illustrated for the gold(I) series **18–21** where the antiplasmodial activity dramatically increases from methyl- (**18**: 22 μM) to mesityl-substituted (**21**: 0.21 nM) systems. By raising the sterically hindrance, the antimalarial activity was highly improved, reflecting an

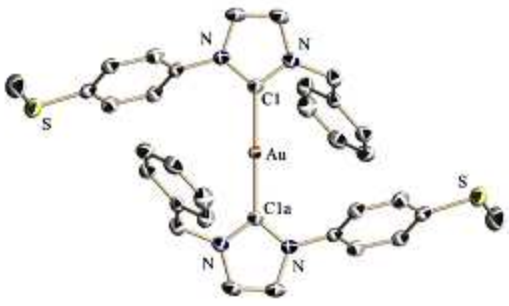



**Figure 2.** Structure of the cationic part of **22** in the solid state, thermal ellipsoids are depicted at a 50% level. Hydrogen atoms and the uncoordinated $PF_6$ anion have been omitted for clarity. Selected bond lengths [Å] and angles [°]: C1–Au = 2.013(2), Au–C1a = 2.013(2), C1–Au–C1a = 180.

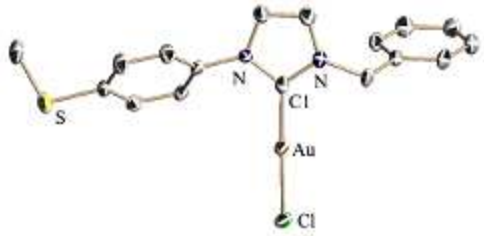



Figure 3. Structure of 23 in the solid state, thermal ellipsoids are depicted at a 50% level. Hydrogen atoms have been omitted for clarity. Selected bond lengths [Å] and angles [°]: C1–Au = 1.972(5), Au–Cl = 2.287(2), C1–Au–Cl = 178.2(2).

increase of the protective effect of the ligand around the metal and probably this allows to avoid releasing of the gold cation before reaching its target.

We have previously reported that the selective cytotoxic activity of this family was directly correlated with the lipophilic properties[8a] and this trend is also verified here: the highest lipophilic complex **21** is the most active against *P. falciparum*. We can hypothesize that lipophilic properties could permit to compounds to better cross the different membranes (red blood cell membrane, parasitophorous vacuole membrane, parasite plasma membrane and eventually digestive vacuole membrane) to target the parasite. Concerning the aromatic nitrogen-containing *N*-functionalized NHC complexes **12**, **14** and **15**, the two quinoline moieties are positioned in trans-arrangement with respect to each other and thus provide already a good protection to the gold(I) center, in contrast to the previous complexes. That could also explain the less antiplasmodial activity with only one quinoline moiety (**13**: 5.2 μM). This feature can account for the same antiplasmodial activity for **12** (1.1 μM) and **14** (1.1 μM), differing in their non-functionalized substituents, methyl and mesityl, respectively. Introducing a second functionalized entity involving a thioether group (**15**) leads to an improvement of the antiproliferative activity with an $IC_{50}$ of 0.47 μM. Moreover, it should be noted that the use of the methylthiophenyl group as the only functionalized group is also very interesting as evidenced by the antimalarial potency of complex **22** ($IC_{50}$ = 0.32 μM).

Compounds **15**, **16**, **20**, **21** and **22** showed thus very high antiplasmodial activity with $IC_{50}$ below the micromolar range. Compounds with the most promising antimalarial properties were also evaluated on culture cell lines in order to determine the specificity of their activity. The selectivity index are ranging from <0.9 to 18. The best selectivity index was found with the compound **21**. These results confirmed those already reported with various cell lines[8a] with $IC_{50}$ values below 1 μM for all cancer cell lines tested whereas the value was superior to 5 μM on non-tumor cells (primary cultures of human umbilical vein endothelial cells (HUVEC)). These data highlight that complex **21** is specifically active against pathogen cells such as *P. falciparum* or multiple cancer cell lines but shows restricted effects on normal human cells.

The potential of organometallic gold complexes without incorporation of known antimalarial organic drugs against parasitic diseases has been little explored until very recently.[3,12] Messori and coll. used a panel of structurally diverse gold(I) and (III) complexes involving N, O or P heterocyclic ligands and the clinically established gold-based antiarthritic drug auranofin, to evaluate them as possible inhibitors of the parasite enzyme Falcipain 2 (Fp2) which is an important protease of *P. falciparum* and represents one of the most promising targets for antimalarial drug discovery.[13] This protein is mainly involved in the degradation of host's hemoglobin (thus providing a substantial amount of peptides and amino acids to sustain parasite growth) and its proteolytic activity is more pronounced at the early trophozoite stage of parasite development. Remarkably, most tested gold compounds caused pronounced but reversible inhibition of Fp2 and important parasite growth inhibition of *P. falciparum* was observed in vitro. However, any direct correlation between enzyme inhibition and reduction of *P. falciparum* growth was established, suggesting that Fp2 inhibition represents just one of the various mechanisms through which gold compounds effectively antagonize *P. falciparum* replication. Chibale and coll. reported gold(I) and gold(III) thiosemicarbazone (TSCs) compounds which exhibit moderate in vitro activity against two *P. falciparum* strains, CQ-sensitive D10 ($IC_{50}$ of CQ = 0.0173 μM) and CQ-resistant W2 ($IC_{50}$ of CQ = 0.095 μM) with $IC_{50}$ ranging from 1.36 to 6.92 μM for the gold(I) compounds and $IC_{50}$ ranging from 3.04 to >20 μM for the gold(III) complexes; the gold(I) TSCs complexes also inhibit the Fp2 but no correlation was found between their antiplasmodial activity and the ability to inhibit Fp2.[14] Mohr and coll. reported gold(I) complexes involving functionalized alkynes that have shown low in vitro activity against falciparum malaria parasite strains CQ-sensitive 3D7 ($IC_{50}$ of CQ = 0.01 μM) and CQ-resistant K1 ($IC_{50}$ of CQ = 0.3 μM) with $IC_{50}$ values between 7.2 and 23 μM.[15] The same authors described also a series of mono- and dinuclear gold(I) phosphine complexes

**Table 1**
Antiplasmodial and cytotoxicity of imidazolium salts and their gold(I) *N*-heterocyclic compounds

| Compounds | Antiplasmodial activity $IC_{50}$ (μM) on *P. falciparum*[*,†] | Cytotoxicity activity $IC_{50}$ (μM) on Vero cell line[†] | Selectivity index |
|---|---|---|---|
| **1** | 17.9 ± 1.3 | | |
| **2** | 1.4 ± 0.2 | >140 | >100 |
| **3** | 1.5 ± 0.1 | 69.7 ± 5.6 | 46 |
| **4** | 73.2 ± 4.5[*] | | |
| **5** | >145[*] | | |
| **6** | >130[*] | | |
| **7** | 59.6 ± 1.4 | | |
| **8** | 11.3 ± 0.6 | | |
| **9** | 6.4 ± 0.1 | | |
| **10** | >230 | | |
| **11** | 9.3 ± 0.3 | | |
| **12**[‖] | 1.1 ± 0.1 | <1[#] | <0.9 |
| **13** | 5.2 ± 2.0 | | |
| **14** | 1.1 ± 0.2 | 6.6 ± 0.4 | 6 |
| **15** | 0.47 ± 0.1 | 3.6 ± 0.3 | 8 |
| **16**[‖] | 0.33 ± 0.03 | 1[#] | 3 |
| **17** | 6.6 ± 0 | | |
| **18** | 2.2 ± 3.8[*] | | |
| **19** | 2.1 ± 0.3[*] | | |
| **20** | 0.48 ± 0.06 | 1.9 ± 0 | 4 |
| **21** | 0.21 ± 0.04 | 3.7 ± 0.1[§] | 18 |
| **22** | 0.32 ± 0.08 | 3.6 ± 0.7 | 11 |
| **23** | 4.1 ± 0.4 | | |
| **24** | 5.6 ± 0.8 | | |
| **25** | 3.7 ± 0.7 | | |
| Chloroquine[*] | 0.514 ± 0.02<br>0.187 ± 0.02[*] | 134 ± 20 | 261<br>717[*] |
| Artemisinin[*] | 0.010 ± 0.7 | ND | |

[*] Antiplasmodial activity of the compounds were evaluated against the *P. falciparum* strain FcM29-Cameroon, except compounds noted (*) evaluated against the *P. falciparum* strain FcB1-Columbia.
[†] Data represented the mean of 2–5 independent experiments.
[‖] Ref. 8c.
[#] Cytotoxicity activity of the compounds were evaluated against Vero cell lines, except compounds noted (#) evaluated on the McF7 line.
[§] Compound **21** was also tested against other cell lines and showed the following results: 0.4 μM (PC-3 cell line); 0.8 μM (MCF-7 cell line); 0.8 μM (U87 cell line); 0.9 μM (A549 cell line); 1 μM (Hep3B cell line); >5 μM (Huvec cell line).[8a]
[*] Both antiplasmodial control drugs artemisinin and chloroquine were routinely tested.

containing mono-anionic seleno- and thiosemicarbazones ligands; the $IC_{50}$ results showed that two sulfur containing mononuclear compounds exhibit activity similar to CQ against the 3D7 strain with $IC_{50}$ of 7.06 and 10.7 nM ($IC_{50}$ of CQ = 8.84 nM).[16] Unfortunately, neither the $IC_{50}$ of the thiosemicarbazones ligands nor the toxicity of the corresponding complexes were evaluated. Previous studies realized by Lambros and co-workers revealed that the two involved ligands themselves already exhibit high in vitro antimalarial activity (CQ-resistant *P. falciparum* Smith strain with $ID_{50}$ below 10 ng/mL ($ID_{90}$ of CQ = 60 ng/mL) and moreover they are toxic in vivo (on *P. berghei* at doses >20 or 40 mg/kg).[17] Cronje and coll. reported preliminary in vitro antimalarial activities of *N*-heterocyclic ylideneamine gold(I) against *P. falciparum*; the coordination of ancillary phosphine or NHC ligands to these complexes results in better activities with an $IC_{50}$ value of 5.1 μg/ml for the NHC derivative.[18] In light of the available literature concerning gold compounds, some of our Au(I)–NHC complexes display improved efficacy.

## 3. Conclusion

In summary, a series of N-, O- or S-functionalized proligands and their corresponding mononuclear cationic, anionic or neutral gold(I) bisNHC complexes, including eight new fully characterized compounds, were tested in vitro for their antimalarial potency against chloroquine-resistant *P. falciparum* strains. Moreover, cytotoxicity of the most active compounds was assessed, in order to determine their selectivity of action. From this screening, the importance of the charge of the molecules tested in the antiplasmodial activity was observed. Cationic lipophilic gold (I)–NHC complexes are more active than similar neutral and anionic complexes. Moreover, an active complex has to be highly lipophilic and the presence of nitrogen or sulfur atoms in the ligands seems to have a positive influence on the antiplasmodial activity.

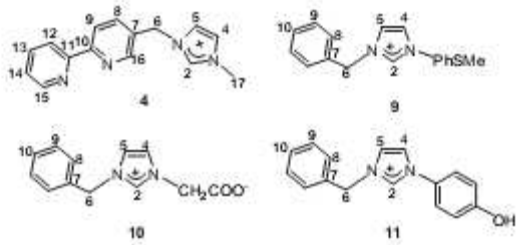
The pharmaco-modulation, by fine tuning either the global charge of the complexes and the key substituents on the NHC ligands, permitted to increase the antiplasmodial activity with promising selectivity indexes and highlighted the interest for gold cationic compounds which can be considered as a good scaffold for the optimization of a new antimalarial chemistry.

## 4. Experimental

### 4.1. Chemistry

#### 4.1.1. General methods

All manipulations were performed under an inert atmosphere of dry nitrogen by using standard vacuum line and Schlenk tube techniques. 3-Methyl-1-{[6-(pyridin-2-yl)pyridin-3-yl]methyl}-1*H*-imidazol-3-ium bromide (**4**), $Ag(4)_2Br$[8c] and 1-(4-methylthiophenyl)-1*H*-imidazole[8d] were synthetized according to literature procedures. All reagents were used as received from commercial suppliers. Reactions involving silver compounds were performed with the exclusion of the light. $^1H$ (300 or 400 MHz) and $^{13}C$ NMR spectra (75 or 101 MHz) were recorded at 298 K on Bruker AV300 or Bruker AV400 spectrometers in $CDCl_3$, $CD_3CN$, DMSO-$d_6$ and $D_2O$ as solvents. Elemental analyses were carried out by the "Service de Microanalyse du Laboratoire de Chimie de Coordination" (Toulouse). High Resolution Mass Spectrometry (HRMS) analysis were performed with a Thermo Finnigan MAT 95 XL spectrometer using electrospray ionization (ESI) or with a TOF mass analyzer by desorption chemical ionization with methane gas (DCI) by the "Service de Spectrométrie de Masse de Chimie UPS-CNRS" (Toulouse).




Numbering of H ($^1H$ NMR) and C ($^{13}C$ NMR). These notations are used in the following section.

#### 4.1.2. 1-(4-Methylthiophenyl)-3-benzylimidazolium chloride (9)

1-(4-Methylthiophenyl)-1*H*-imidazole (1.00 g, 5.26 mmol), benzylchloride (605 μL, 5.26 mmol) and $KPF_6$ (967 mg, 5.26 mmol) were stirred in $CH_3CN$ (20 mL) at 80 °C for 3 days. After cooling to room temperature, the solvent was evaporated and the yellow solid obtained was washed with $CH_2Cl_2$ and diethylether and dried under vacuum (2.018 g, 90% yield). Anal. Calcd for $C_{17}H_{17}N_2SPF_6$: C, 47.89; H, 4.02; N, 6.57. Found: C, 47.93; H, 4.06; N, 6.55. $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.44 (s, 1H, $H_2$), 8.38 (d, $J$ = 1.6 Hz, 1H, $H_5$), 8.11 (d, $J$ = 1.6 Hz, 1H, $H_4$), 7.79 (d, $J$ = 8.6 Hz, 2H, $H_{PhSMe}$), 7.99 (d, $J$ = 6.6 Hz, 2H, $H_8$), 7.49 (d, $J$ = 8.6 Hz, 2H, $H_{PhSMe}$), 7.45–7.37 (m, 3H, $H_9$, $H_{10}$), 5.57 (s, 2H, $H_6$), 2.54 (s, 3H, $H_{PhSMe}$). $^{13}C$ NMR (75 MHz, DMSO-$d_6$) $\delta$ 141.37 (1C, $C_{PhSMe}$), 135.73 (1C, $C_2$), 134.79 (1C, $C_7$), 131.93 (1C, $C_{PhSMe}$), 129.47 (2C, $C_9$), 129.34 (1C, $C_{10}$), 128.95 (2C, $C_8$), 127.09 (2C, $C_{PhSMe}$), 123.58 (1C, $C_5$), 122.86 (2C, $C_{PhSMe}$), 122.12 (1C, $C_4$), 52.93 (1C, $C_6$), 14.92 (1C, $C_{PhSMe}$). HRMS (ES$^+$) calculated for $C_{17}H_{17}N_2S(M-PF_6)^+$ 281.1112, found 281.1120.

#### 4.1.3. 1-(Carboxymethyl)-3-benzylimidazolium (10)

Benzylimidazole (419 mg, 2.65 mmol) and bromoacetic acid (368 mg, 2.65 mmol) were heated at 130 °C for 24 h. After the mixture was cooled to room temperature, the oily product was dissolved in 20 ml $H_2O$ and $Na_2CO_3$ was added (281 mg, 2.65 mmol). The mixture was refluxed at 100 °C for 30 min. After evaporation of the solvent, the resulting solid was partially dissolved in MeOH, filtered and the filtrate was evaporated to dryness. The crude product was washed with $CH_2Cl_2$ and dried under vacuum to afford a white solid (0.44 g, 77% yield). Anal. Calcd for $C_{12}H_{12}N_2O_2$: C, 66.65; H, 5.59; N, 12.96. Found: C, 66.58; H, 5.66; N, 12.90. $^1H$ NMR (300 MHz, DMSO-$d_6$) $\delta$ 9.21 (s, 1H, $H_2$), 7.69 (s, 1H, $H_5$), 7.60 (s, 1H, $H_4$), 7.40 (m, 5H, $H_8$, $H_9$, $H_{10}$), 5.43 (s, 2H, $H_6$), 4.45 (s, 2H, $H_{CH2COO}$). $^{13}C$ NMR (75 MHz, $D_2O$) $\delta$ 172.10 (1C, $C_{COO}$), 163.97 (1C, $C_2$), 133.44 (1C, $C_7$), 129.36 (2C, $C_9$), 129.31 (1C, $C_{10}$), 128.70 (2C, $C_8$), 123.71 (1C, $C_5$), 121.94 (1C, $C_4$), 52.89 (1C, $C_6$), 52.01 (1C, $C_{CH2COO}$). HRMS (ES$^+$) calculated for $C_{12}H_{13}N_2O_2$ (M+H)$^+$ 217.0977, found 217.0978.

#### 4.1.4. 1-(4-Hydroxyphenyl)-3-benzylimidazolium hexafluorophosphate (11)

1-(4-Hydroxyphenyl)imidazole (1.642 g, 9.760 mmol), benzylchloride (1.15 mL, 9.760 mmol) and $KPF_6$ (1.800 g, 9.760 mmol) were heated in acetonitrile (20 mL) at 80 °C for 3 days. The white solid obtained was filtered and dried under vacuum (3.595 g, 93% yield). Anal. Calcd for $C_{16}H_{15}N_2OPF_6$: C, 48.50; H, 3.82; N, 7.07. Found: C, 48.47; H, 3.95; N, 7.01. $^1H$ NMR (300 MHz, DMSO-$d_6$) $\delta$ 10.31 (s, 1H, $H_{PhOH}$), 9.74 (t, $J$ = 1.8 Hz, 1H, $H_2$), 8.11 (t, $J$ = 1.9 Hz, 1H, $H_5$), 7.90 (t, $J$ = 1.9 Hz, 1H, $H_4$), 7.56 (d, $J$ = 8.9 Hz, 2H, $H_{PhOH}$), 7.53–7.36 (m, 5H, $H_8$, $H_9$, $H_{10}$), 6.98 (d, $J$ = 8.9 Hz, 2H, $H_{PhOH}$), 5.47 (s, 2H, $H_6$). $^{13}C$ NMR (75 MHz, DMSO-$d_6$) $\delta$ 159.09 (1C, $C_{PhOH}$),

135.58 (1C, $C_2$), 135.01 (1C, $C_7$), 129.49 (2C, $C_9$), 129.32 (1C, $C_{10}$), 128.89 (2C, $C_8$), 126.94 (1C, $C_{PhOH}$), 124.23 (2C, $C_{PhOH}$), 123.43 (1C, $C_5$), 122.52 (1C, $C_4$), 116.74 (2C, $C_{PhOH}$), 52.83 (1C, $C_6$). HRMS (ES$^+$) calculated for $C_{16}H_{15}N_2O$ (M−$PF_6$)$^+$ 251.1184, found 251.1189.

#### 4.1.5. {1-[([2,2′-Bipyridin]-5-yl)methyl]-3-methylimidazol-2-ylidene}(chloro)gold(I) (17)

Complex **17** was obtained by transmetalation of $Ag(4)_2Br$ (81 mg, 0.118 mmol) with $Au(SMe_2)Cl$ (69.2 mg, 0.235 mmol) in a mixture of $CH_2Cl_2$–MeOH (4 mL / 2 mL) at rt. After stirring for 1 h, the solution was filtered through a pad of celite and the solvent removed under reduced pressure to give a gray solid (0.72 g, 63% yield). Anal. Calcd for $C_{15}H_{14}N_4AuCl$: C, 37.32; H, 2.92; N, 11.61. Found: C, 37.27; H, 3.06; N, 11.57. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.73–8.67 (m, 2H, $H_{15}$, $H_{16}$), 8.48 (d, $J$ = 8.2 Hz, 1H, $H_{12}$), 8.43 (d, $J$ = 8.0 Hz, 1H, $H_9$), 7.95–7.80 (m, 2H, $H_8$, $H_{13}$), 7.39 (ddd, $J$ = 7.5, 4.8, 1.1 Hz, 1H, $H_{14}$), 7.00 (d, $J$ = 1.9 Hz, 1H, $H_5$), 6.96 (d, $J$ = 1.9 Hz, 1H, $H_4$), 5.49 (s, 2H, $H_6$), 3.90 (s, 3H, $H_{17}$). $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 171.99 (1C, $C_2$), 156.78 (1C, $C_{10}$), 155.25 (1C, $C_{11}$), 149.25 (1C, $C_{15}$), 148.50 (1C, $C_{16}$), 137.05 (1C, $C_{13}$), 136.68 (1C, $C_8$), 130.61 (1C, $C_7$), 124.11 (1C, $C_{14}$), 122.70 (1C, $C_5$), 121.31 (1C, $C_9$), 121.25 (1C, $C_{12}$), 120.21 (1C, $C_4$), 52.41 (1C, $C_6$), 38.41 (1C, $C_{17}$). HRMS (ES$^+$) calculated for $C_{15}H_{14}N_4Au$ (M−Cl)$^+$ 447.0884, found 447.0887.

#### 4.1.6. Bis{1-benzyl-3-[4-(methylsulfanyl)phenyl]imidazol-2-ylidene}gold(I) hexafluorophosphate (22)

Under a nitrogen atmosphere, sodium acetate (69.3 mg, 0.844 mmol) was added to a mixture of **9** (300 mg, 0.704 mmol) and $Au(SMe_2)Cl$ (104 mg, 0.352 mmol) in dry DMF (6 mL) at 100 °C. The mixture was then heated to 120 °C and this temperature was maintained for 2 h. After cooling to room temperature, the solution was filtered through a pad of celite and the solvent removed under reduced pressure to give a white solid (0.277 g, 87% yield). Anal. Calcd for $C_{34}H_{32}N_4S_2PF_6Au$: C, 45.24; H, 3.57; N, 6.21. Found: C, 45.29; H, 3.62; N, 6.24. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.88 (d, $J$ = 1.9 Hz, 2H, $H_5$), 7.82 (d, $J$ = 1.9 Hz, 2H, $H_4$), 7.63 (d, 4H, $J$ = 8.6 Hz, $H_{PhSMe}$), 7.37–7.28 (m, 6H, $H_9$, $H_{10}$), 7.26 (d, 4H, $J$ = 8.6 Hz, $H_{PhSMe}$), 7.24–7.17 (m, 4H, $H_8$), 5.35 (s, 4H, $H_6$), 2.45 (s, 6H, $H_{PhSMe}$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 181.57 (2C, $C_2$), 140.26 (2C, $C_{PhSMe}$), 136.62 (2C, $C_7$), 135.92 (2C, $C_{PhSMe}$), 129.21 (4C, $C_9$), 128.66 (2C, $C_{10}$), 127.94 (4C $C_8$), 126.64 (4C, $C_{PhSMe}$), 125.71 (4C, $C_{PhSMe}$), 123.93 (2C, $C_5$), 123.61 (2C, $C_4$), 54.35 (2C, $C_6$), 14.95 (2C, $C_{PhSMe}$). HRMS (ES$^+$) calculated for $C_{34}H_{32}N_4S_2Au$ (M−$PF_6$)$^+$ 757.1734, found 757.1744.

#### 4.1.7. {1-Benzyl-3-[4-(methylsulfanyl)phenyl]imidazol-2-ylidene}(chloro)gold(I) (23)

Under a nitrogen atmosphere and protection of the light, a Schlenk tube was charged with **9** (261 mg, 0.612 mmol) in a mixture of $CH_2Cl_2$–MeOH (5 mL/1 mL), $Ag_2O$ (71 mg, 0.306 mmol) and KCl (68 mg, 0.918 mmol). The reaction mixture was stirred at rt for 6 h. After that $Au(SMe_2)Cl$ (180 mg, 0.612 mmol) was added and the mixture was stirred for 4 h. The solution was filtered through a pad of celite and the solvent removed under reduced pressure to give a gray solid (0.267 g, 82% yield). Anal. Calcd for $C_{17}H_{16}N_2SClAu$: C, 39.82; H, 3.15; N, 5.46. Found: C, 39.76; H, 3.22; N, 5.48. $^1$H NMR (300 MHz, $CD_3CN$) $\delta$ 7.63 (d, $J$ = 8.8 Hz, 2H, $H_{PhSMe}$), 7.48–7.38 (m, 8H, $H_{PhSMe}$, $H_8$, $H_9$, $H_{10}$), 7.36 (d, $J$ = 2.0 Hz, 1H, $H_4$), 5.48 (s, 2H, $H_6$), 2.56 (s, 3H, $H_{PhSMe}$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 168.90 (1C, $C_2$), 140.10 (1C, $C_{PhSMe}$), 136.84 (1C, $C_7$), 136.18 (1C, $C_{PhSMe}$), 129.30 (2C, $C_9$), 128.69 (1C, $C_{10}$), 128.23 (2C, $C_8$), 126.73 (2C, $C_{PhSMe}$), 125.93 (2C, $C_{PhSMe}$), 123.57 (1C, $C_5$), 123.01 (1C, $C_4$), 54.62 (1C, $C_6$), 15.00 (1C, $C_{PhSMe}$). HRMS (DCI) calculated for $C_{17}H_{16}N_2SAu$ (M−Cl)$^+$ 477.0700, found 477.0710.

#### 4.1.8. Hydrogen bis[1-benzyl-3-(carboxylatomethyl)imidazol-2-ylidene]gold(I) (24)

Under a nitrogen atmosphere and protection of the light, a Schlenk tube was charged with **10** (338 mg, 1.563 mmol) in 10 ml of dry MeOH and $Ag_2O$ (181 mg, 0.782 mmol). The mixture was stirred for 12 h at 50 °C. After cooling to rt, $Au(SMe_2)Cl$ (230 mg, 0.782 mmol) was added and the mixture was stirred at rt for 2 h. The solution was filtered through a pad of celite and the solvent removed under reduced pressure to give a gray solid (0.407 g, 83% yield). Anal. Calcd for $C_{24}H_{23}N_4O_4Au$: C, 45.87; H, 3.69; N, 8.92. Found: C, 45.90; H, 3.90; N, 8.93. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.45 (s, 2H, $H_5$), 7.39–7.28 (m, 12H, $H_4$, $H_8$, $H_9$, $H_{10}$), 5.32 (s, 4H, $H_6$), 4.50 (s, 2H, $H_{CH2COO}$), 4.42 (s, 2H, $H_{CH2COO}$). $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ 184.51 (2C, $C_2$), 174.02 (2C, $C_{COO}$), 136.53 (2C, $C_7$), 128.83 (4C, $C_9$), 128.15 (2C, $C_{10}$), 127.40 (4C, $C_8$), 123.05 (2C, $C_5$), 121.74 (2C, $C_4$), 53.91 (2C, $C_6$), 53.85 (2C, $C_{CH2COO}$). HRMS (ES$^+$) calculated for $C_{24}H_{24}N_4O_4Au$ (M+H)$^+$ 629.1463, found 629.1478.

#### 4.1.9. Hydrogen bis[1-benzyl-3-[(4-oxidophenyl)]imidazol-2-ylidene]gold(I) (25)

Under a nitrogen atmosphere, sodium acetate (74.5 mg, 0.908 mmol) was added to a mixture of **11** (300 mg, 0.757 mmol) and $Au(SMe_2)Cl$ (112 mg, 0.379 mmol) in dry DMF (5 mL) at 100 °C. The mixture was then heated to 120 °C and this temperature was maintained for 2 h. After cooling to room temperature, $CH_2Cl_2$ (5 mL) and hexane (10 mL) were added to the solution to afford a beige solid, which was filtered and dried under vacuum (0.175 g, 66% yield). Anal. Calcd for $C_{32}H_{27}N_4O_2Au$: C, 55.18; H, 3.91; N, 8.04. Found: C, 55.23; H, 3.96; N, 8.10. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.77–7.76 (m, 4H, $H_4$, $H_5$), 7.37 (d, $J$ = 8.8 Hz, 4H, $H_{PhO}$), 7.31–7.29 (m, 6H, $H_9$, $H_{10}$), 7.19 (dd, $J$ = 6.7, 2.9 Hz, 4H, $H_8$), 6.82 (d, $J$ = 8.8 Hz, 4H, $H_{PhO}$), 5.31 (s, 4H, $H_6$). $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 181.55 (2C, $C_2$), 158.36 (2C, $C_{PhO}$), 136.66 (2C, $C_7$), 130.71 (2C, $C_{PhO}$), 129.22 (4C, $C_9$), 128.69 (2C, $C_{10}$), 127.94 (4C, $C_8$), 126.43 (4C, $C_{PhO}$), 123.88 (2C, $C_5$), 123.12 (2C, $C_4$), 116.24 (4C, $C_{PhO}$), 54.19 (2C, $C_6$). HRMS (ES$^+$) calculated for $C_{32}H_{28}N_4O_2Au$ (M+H)$^+$ 697.1878, found 697.1874.

### 4.2. Crystallographic data for 17, 22 and 23

All data were collected at low temperature using oil-coated shock-cooled crystals on a Bruker-AXS APEX II diffractometer with MoK$\alpha$ radiation ($\lambda$ = 0.71073 Å). The structures were solved by direct methods[19] and all non hydrogen atoms were refined anisotropically using the least-squares method on $F^2$.[20]

CCDC-1441512 (**17**), CCDC-1441513 (**22**) and CCDC-1441514 (**23**) contain the supplementary crystallographic data. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

**17**: $C_{15}H_{14}AuClN_4$, Mr = 482.72, crystal size = 0.30 × 0.30 × 0.10 mm$^3$, monoclinic, space group $P2_1/c$, $a$ = 5.6934(3) Å, $b$ = 11.8070(6) Å, $c$ = 21.8435(12) Å, $\beta$ = 92.646(3)°, $V$ = 1466.8 (2) Å$^3$, $Z$ = 4, $T$ = 173(2) K, 2506 unique reflections, $R_1$ = 0.0554, $wR_2$ = 0.1535 [$I > 2\sigma(I)$], $R_1$ = 0.0759, $wR_2$ = 0.1659 (all data), residual electron density = 2.400 e Å$^{-3}$.

**22**: $C_{34}H_{32}AuF_6N_4PS_2$, Mr = 902.69, crystal size = 0.30 × 0.30 × 0.10 mm$^3$, monoclinic, space group $P2_1/n$, $a$ = 11.7142(5) Å, $b$ = 11.6965(5) Å, $c$ = 12.7877(6) Å, $\beta$ = 108.408(1)°, $V$ = 1662.5 (2) Å$^3$, $Z$ = 2, $T$ = 173(2) K, 24252 reflections collected, 6058 unique reflections ($R_{int}$ = 0.0409), $R_1$ = 0.0228, $wR_2$ = 0.0551 [$I > 2\sigma(I)$], $R_1$ = 0.0324, $wR_2$ = 0.0613 (all data), residual electron density = 1.811 e Å$^{-3}$.

**23**: $C_{17}H_{16}AuClN_2S$, Mr = 512.79, crystal size = 0.30 × 0.20 × 0.05 mm$^3$, triclinic, space group $P\bar{1}$, $a$ = 9.545(1) Å, $b$ = 9.931(1) Å, $c$ = 10.044(1) Å, $\alpha$ = 103.547(4)°, $\beta$ = 112.197(4)°,

$\gamma = 97.717(4)°$, $V = 830.4(2)$ Å$^3$, $Z = 2$, $T = 173(2)$ K, 11653 reflections collected, 4054 unique reflections ($R_{int} = 0.0366$), $R_1 = 0.0276$, $wR_2 = 0.0679$ [$I > 2\sigma(I)$], $R_1 = 0.0320$, $wR_2 = 0.0702$ (all data), residual electron density = 2.108 e Å$^{-3}$.

### 4.3. Biological material and methods

#### 4.3.1. In vitro antimalarial activities

The in vitro antimalarial activities of the 25 synthesized compounds were tested comparatively to their ligands in order to determine the influence of the different structure modulations on the biological activities. Both chloroquine-resistant *P. falciparum* strains FcM29-Cameroon and FcB1-Colombia, were cultured continuously according to the modified Trager and Jensen's method.[21] The antimalarial activity was evaluated by the radioactive micromethod described by Desjardins et al.[22] with modifications as reported.[23]

Drug testing was performed from 2 to 5 times in triplicate in 96-well culture plates on D-sorbitol synchronized cultures. Compounds were dissolved in dimethylsulfoxide (DMSO) (stock solution: 10 mg/mL) and further diluted in culture medium so that the final DMSO concentration never exceeded 2%. For each assay, it was verified that the 2% concentration of DMSO did not affect parasite growth. Parasite growth was estimated by [$^3$H]hypoxanthine incorporation (Perkin Elmer, France).

The control parasite culture (culture medium with or without 2% DMSO) was referred to as 100% growth. The $IC_{50}$ values were determined graphically by plotting the log of the drug concentration versus the percentage inhibition of parasite growth. Results are summarized in Table 1.

#### 4.3.2. Cytotoxicity assays

Evaluation of the cytotoxicity of selected compounds was performed on Vero cell lines (Monkey epithelial cell line, Sigma–Aldrich) or on McF7 cell line with the same dilution protocol used for antiplasmodial assays. Cell lines were distributed in 96-well plates in 100 μL MEM medium with 10% fetal calf serum at 37 °C under a humidified atmosphere (5% $CO_2$). After 24 h-culture, the cells were supplemented with 100 μL of the compounds dilutions and [$^3$H]hypoxanthine was also added for 48 h more. Cell growth was estimated by [$^3$H]hypoxanthine incorporation read by β counter (1450-Microbeta Trilux, Wallac-Perkin Elmer) as reported for antiplasmodial assays.[24] Selectivity index was then calculated by the cytotoxicity/activity ratio.

### Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique (CNRS), France. APR obtained a PhD grant from DIKTI—Indonesia.

### Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.05.023.

### References and notes

1. WHO, World Health Organization. World Malaria Report 2015. **2015**, p. 280.
2. Alessio, E., Bioinorg. Med. Chem. Germany **2011**; Vol. 10.
3. Salas, P. F.; Herrmann, C.; Orvig, C. *Chem. Rev.* **2013**, *113*, 3450.
4. (a) Navarro, M.; Castro, W.; Martínez, A.; Sánchez-Delgado, R. *J. Inorg. Biochem.* **2011**, *105*, 276; (b) Martinez, A.; Rajapakse, C. S. K.; Jalloh, D.; Dautriche, C.; Sanchez-Delgado, R. A. *J. Biol. Inorg. Chem.* **2009**, *14*, 863; (c) Navarro, M.; Vasquez, F.; Sanchez-Delgado, R. A.; Perez, H.; Sinou, V.; Schrevel, J. *J. Med. Chem.* **2004**, *47*, 5204; (d) Sánchez-Delgado, R.; Navarro, M.; Pérez, H. *J. Chem. Med.* **1997**, *40*, 1937; (e) Sanchez-Delgado, R. A.; Navarro, M.; Perez, H.; Urbina, J. A. *J. Med. Chem.* **1996**, *39*, 1095.
5. (a) Biot, C.; Glorian, G.; Maciejewski, L. A.; Brocard, J. S.; Domarle, O.; Blampain, G.; Millet, P.; Georges, A. J.; Abessolo, H.; Dive, D.; Lebibi, J. *J. Med. Chem.* **1997**, *40*, 3715; (b) Chavain, N.; Vezin, H.; Dive, D.; Touati, N.; Paul, J. F.; Buisine, E.; Biot, C. *Mol. Pharm.* **2008**, *5*, 710; (c) Biot, C.; Nosten, F.; Fraisse, L.; Ter-Minassian, D.; Khalife, J.; Dive, D. *Parasite* **2011**, *18*, 207; (d) Biot, C.; Pradines, B.; Dive, D. *Drug Discov. Infect. Dis.* **2011**; (e) Dubar, F.; Bohic, S.; Slomianny, C.; Morin, J. C.; Thomas, P.; Kalamou, H.; Guerardel, Y.; Cloetens, P.; Khalife, J.; Biot, C. *Chem. Commun.* **2012**, 910; (f) ClinicalTrials.gov, http://clinicaltrials.gov/ct2/show/NCT00988507 & https://clinicaltrials.gov/ct2/show/NCT02497612.
6. Chavain, N.; Davioud-Charvet, E.; Trivelli, X.; Mbeki, L.; Rottmann, M.; Brun, R.; Biot, C. *Bioorg. Med. Chem.* **2009**, *17*, 8048.
7. (a) Teyssot, M. L.; Jarrousse, A. S.; Manin, M.; Chevry, A.; Roche, S.; Norre, F.; Beaudoin, C.; Morel, L.; Boyer, D.; Mahiou, R.; Gautier, A. *Dalton Trans.* **2009**, 6894; (b) Hindi, K. M.; Panzner, M. J.; Tessier, C. A.; Cannon, C. L.; Youngs, W. J. *Chem. Rev.* **2009**, *109*, 3859; (c) John, A.; Ghosh, P. *Dalton Trans.* **2010**, *39*, 7183; (d) Mercs, L.; Albrecht, M. *Chem. Soc. Rev.* **2010**, *39*, 1903; (e) Gasser, G.; Ott, I.; Metzler-Nolte, N. *J. Med. Chem.* **2011**, *54*, 3; (f) Gautier, A.; Cisnetti, F. *Metallomics* **2012**, *4*, 23; (g) Patra, M.; Gasser, G.; Metzler-Nolte, N. *Dalton Trans.* **2012**, *41*, 6350; (h) Liu, W. K.; Gust, R. *Chem. Soc. Rev.* **2013**, *42*, 755; (i) Oehninger, L.; Rubbiani, R.; Ott, I. *Dalton Trans.* **2013**, *42*, 3269; (j) Bertrand, B.; Casini, A. *Dalton Trans.* **2014**, *43*, 4209; (k) Tacke, M. *J. Organomet. Chem.* **2015**, *782*, 17.
8. (a) Boselli, L.; Ader, I.; Carraz, M.; Hemmert, C.; Cuvillier, O.; Gornitzka, H. *Eur. J. Med. Chem.* **2014**, *85*, 87; (b) Boselli, L.; Carraz, M.; Mazeres, S.; Paloque, L.; Gonzalez, G.; Benoit-Vical, F.; Valentin, A.; Hemmert, C.; Gornitzka, H. *Organometallics* **2015**, *34*, 1046; (c) Hemmert, C.; Fabie, A.; Fabre, A.; Benoit-Vical, F.; Gornitzka, H. *Eur. J. Med. Chem.* **2013**, *60*, 64; (d) Paloque, L.; Hemmert, C.; Valentin, A.; Gornitzka, H. *Eur. J. Med. Chem.* **2015**, *94*, 22.
9. Lin, J. C. Y.; Huang, R. T. W.; Lee, C. S.; Bhattacharyya, A.; Hwang, W. S.; Lin, I. J. B. *Chem. Rev.* **2009**, *109*, 3561.
10. Wein, S.; Maynadier, M.; Bordat, Y.; Perez, J.; Maheshwari, S.; Bette-Bobillo, P.; Tran Van Ba, C.; Penarete-Vargas, D.; Fraisse, L.; Cerdan, R.; Vial, H. *Br. J. Pharmacol.* **2012**, *166*, 2263.
11. Kuhn, Y.; Rohrbach, P.; Lanzer, M. *Cell Microbiol.* **2007**, *9*, 1004.
12. Glisic, B. D.; Djuran, M. I. *Dalton Trans.* **2014**, *43*, 5950.
13. Micale, N.; Cinellu, M. A.; Maiore, L.; Sannella, A. R.; Severini, C.; Schirmeister, T.; Gabbiani, C.; Messori, L. *J. Inorg. Biochem.* **2011**, *105*, 1576.
14. (a) Khanye, S. D.; Wan, B.; Franzblau, S. G.; Gut, J.; Rosenthal, P. J.; Smith, G. S.; Chibale, K. *J. Org. Chem.* **2011**, *696*, 3392; (b) Khanye, S. D.; Smith, G. S.; Lategan, C.; Smith, P. J.; Gut, J.; Rosenthal, P. J.; Chibale, K. *J. Inorg. Biochem.* **2010**, *104*, 1079.
15. Schuh, E.; Valiahdi, S. M.; Jakupec, M. A.; Keppler, B. K.; Chiba, P.; Mohr, F. *Dalton Trans.* **2009**, 10841.
16. Molter, A.; Rust, J.; Lehmann, C. W.; Deepa, G.; Chiba, P.; Mohr, F. *Dalton Trans.* **2011**, *40*, 9810.
17. Lambros, C.; Childs, G. E.; Notsch, J. D.; Scovill, J. P.; Klayman, D. L.; Davidson, D. E. *Antimicrob. Agents Chemother.* **1982**, *22*, 981.
18. Coetzee, J.; Cronje, S.; Dobrzanska, L.; Raubenheimer, H. G.; Joone, G.; Nell, M. J.; Hoppe, H. C. *Dalton Trans.* **2011**, *40*, 1471.
19. Sheldrick, G. M. *Acta Crystallogr.* **1990**, *A46*, 467.
20. Sheldrick, G. M. *Acta Crystallogr.* **2008**, *A64*, 112.
21. Benoit-Vical, F.; Robert, A.; Meunier, B. *Antimicrob. Agents Chemother.* **2000**, *44*, 2836.
22. Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. *Antimicrob. Agents Chemother.* **1979**, *16*, 710.
23. Benoit-Vical, F.; Lelievre, J.; Berry, A.; Deymier, C.; Dechy-Cabaret, O.; Cazelles, J.; Loup, C.; Robert, A.; Magnaval, J. F.; Meunier, B. *Antimicrob. Agents Chemother.* **2007**, *51*, 1463.
24. Benoit-Vical, F.; Soh, P. N.; Salery, M.; Harguem, L.; Poupat, C.; Nongonierma, R. *J. Ethnopharmacol.* **2008**, *116*, 43.

## C. Gene *Pfk13* and Artemisinin Resistance

### a. Results

Based on the identification of a candidate marker of artemisinin resistance from the highly artemisinin resistant laboratory strain F32-ART, a study conducted by Ariey *et al.* led to precision of the molecular marker of this resistance in isolated resistant parasites from patients in Cambodia. It was demonstrated that specific mutations in the K13-propeller are strongly correlated with artemisinin resistance, both *in vitro* and *in vivo*. Three-high frequency mutant alleles with high survival rates determined by $RSA_{0\text{-}3h}$ wereY493H, R539T, and C580Y [24] and long parasite clearance half-lives (>5 hours) in patients [207]. On the other hand, first K13 mutant allele that have been observed was M476I. It was found in Tanzanian F32 parasites that experienced long-term artemisinin pressure *in vitro* for over 5 years, yielding the F32-ART line [22].

Therefore, the molecular mechanism of this resistance would be defined by determining the impact of *Pfk13* gene mutations on the levels of artemisinin resistance in clinical isolates in lab strains and assessing the role of individual polymorphism in certain types of genetic backgrounds. The study was a collaborative work between CNRS-LCC- (Dr F. Benoit-Vical, Toulouse, France), Columbia University College of Physicians and Surgeons (Dr D. Fidock, New York, USA), Institut Pasteur Paris (Dr O. Mercereau-Puijalon, France), and Institut Pasteur du Cambodge (Dr D. Ménard, Cambodia).

Zinc-finger nucleases (ZFNs) were successfully used to modify K13 genetically culture-adapted Cambodian isolates and the well-known reference lines of *P. falciparum* (Dd2, FcB, V1/S, F32-ART). ZFNs promoted double-stranded break in K13 genomic target locus followed by DNA resection and repair, allowing to capture the mutations transferred by $pZFN^{K13}$-*hdhfr* plasmids.

Assessment of mutations insertion or removal was conducted through $RSA_{0\text{-}3h}$ assay on parenteral and edited parasites. The 0-3 hours of post-invasion ring (1% parasitemia, 2% hematocrit) were exposed to 700 nM Dihydroartemisinin (DHA) for 6 hours. To remove drug, a washing step was employed by adding culture medium and followed by returning fresh medium without drug to culture conditions for 66 hours. The survival rate was determined by assessing parasite viability using microscopic examination of Giemsa-stained thin blood smears.

Higher survival rates were obtained from Cambodian isolates ($Cam3.I^{R539T}$ and $Cam3.II^{R539T}$) containing the R539T mutation. In contrast, significantly lower survival rates were found when the R539T mutation was removed from the isolates, restoring therefore the wild type allele ($Cam3.I^{rev}$ and $Cam3.II^{rev}$). Same results were observed with I543T mutation removal (43% survival rates in $Cam5^{I5435T}$ versus 0.3% in $Cam5^{rev}$) and C580Y mutation removal (13% survival rates in $Cam2^{C580Y}$ versus 2.4% in $Cam2^{rev}$).

Moreover, survival rates increased when K13 mutation was introduced to wild type carrying parasite (CamWT, $Cam3.II^{rev}$, V1/S, F32-TEM and FcB). In details, C580Y to CamWT (0.6% survival rate in CamWT to 8.9% in $CamWT^{C580Y}$), $Cam3.II^{rev}$ and C580Y (0.7% in $Cam3.II^{rev}$ and 24.1% in $Cam3.II^{C580Y}$), R539T to V1/S (0.3% in V1/S versus 20.7% in $V1/S^{R539T}$) showed significant increase, while it was moderate with M476I for F32-TEM and C580Y for FcB.

To examine the differences of K13 mutations in ART resistance level, M476I, R539T, I543T were inserted in the Dd2 reference line resulting in higher survival rates than when Y493H and C580Y were introduced. Although C580Y mutation was predominant in Cambodia, Laos and Vietnam [28], the associated survival rates increased modestly. The variety of survival rates obtained after the insertion of the mutation C580Y into different clones

like Dd2, FcB, and three Cambodian isolates indicated the influence of the parasite genetic background. This phenomenon was also found in other Asian countries, but not in the most endemic malaria region, Africa, because the Cambodian parasite has restricted genetic admixture that contains distinctive K13 mutation as opposed to the one in Africa.

Latest publications determined that non-synonymous K13 mutations were located in Southeast Asia to China with proportion ranging from moderate (10-20%) to fixed (> 95%), and were confirmed to be associated with artemisinin resistance. Based on geographical localization, there are two areas harboring specific individual mutations including Cambodia, Vietnam, and Laos for C580Y, R539T, Y493H, and I543T and Thailand, Myanmar, and China for F446I, N458Y, P574L, and R561H. K13 mutations were uncommon in Africa, but were thought to be found in few African countries (the proportion ranging from 3.0% to 8.3%). Further identifications showed that there was no Asian K13 mutation allele in African samples. Moreover, the fourth most frequent mutant allele in Africa observed in one sample from Thailand, A578S, was clearly defined as not correlated to artemisinin-resistance. It is suggested that for the moment, no ART-resistance was reported in Africa. Furthermore, no Asian ART-resistant alleles were found in Africa isolates [28].

It has been demonstrated that K13-propeller mutations lead to ART resistance *in vitro* and are a relevant marker of this resistance in Asia. Hence, it is worth considering that global sequencing effort for K13 is conducted to trace the distribution of ART resistance and to initiate the impact mitigation for supporting world malaria elimination program, especially in highest incidence level area in Africa. Furthermore, K13 mutations monitoring is included in the WHO criteria to follow ART resistance worldwide.

b. **Corresponding publication**




9. Materials and methods are available as supplementary materials on Science Online.
10. R. C. Burns, L. A. Devereux, P. Granger, G. J. Schrobilgen, *Inorg. Chem.* 24, 2615–2624 (1985).
11. C.-W. Park, R. J. Salm, J. A. Ibers, *Can. J. Chem.* 73, 1148–1156 (1995).
12. S. Bag, P. N. Trikalitis, P. J. Chupas, G. S. Armatas, M. G. Kanatzidis, *Science* 317, 490–493 (2007).
13. M. V. Kovalenko, M. Scheele, D. V. Talapin, *Science* 324, 1417–1420 (2009).
14. D. V. Talapin, C. B. Murray, *Science* 310, 86–89 (2005).
15. D. S. Chung *et al.*, *Nano Lett.* 12, 1813–1820 (2012).
16. Y. Liu *et al.*, *Nano Lett.* 10, 1960–1969 (2010).
17. Y. Liu *et al.*, *Nano Lett.* 13, 1578–1587 (2013).
18. J. H. Choi *et al.*, *Nano Lett.* 12, 2631–2638 (2012).
19. J. S. Lee, M. V. Kovalenko, J. Huang, D. S. Chung, D. V. Talapin, *Nat. Nanotechnol.* 6, 348–352 (2011).
20. D. Yu, C. Wang, P. Guyot-Sionnest, *Science* 300, 1277–1280 (2003).
21. S. S. Devlin, *Physics and Chemistry of II-VI Compounds* (North Holland, Amsterdam, 1967).
22. B. M. Basol, S. S. Ou, O. M. Stafsudd, *J. Appl. Phys.* 58, 3809–3813 (1985).
23. S. J. Oh *et al.*, *Nano Lett.* 14, 1559–1566 (2014).
24. B. A. Ridley, B. Nivi, J. M. Jacobson, *Science* 286, 746–749 (1999).
25. W. J. Baumgardner, K. Whitham, T. Hanrath, *Nano Lett.* 13, 3225–3231 (2013).
26. Z. M. Norman, N. C. Anderson, J. S. Owen, *ACS Nano* 8, 7513–7521 (2014).
27. S. N. Girard *et al.*, *J. Am. Chem. Soc.* 133, 16588–16597 (2011).
28. K. Biswas *et al.*, *Nature* 489, 414–418 (2012).
29. R. Klajn *et al.*, *Science* 316, 261–264 (2007).
30. S.-J. L. Kang, *Sintering: Densification, Grain Growth and Microstructure* (Butterworth-Heinemann, Oxford, 2004).



**ACKNOWLEDGMENTS**

We thank S. Kwon and V. Zyryanov for help with EXAFS measurements and N. James for reading the manuscript. The work was supported by the II-VI Foundation, U.S. Department of Energy (DOE) SunShot program under award DE-EE0005312 and by NSF under award DMR-1310398. D.V.T. also thanks the Keck Foundation. This work used facilities supported by the NSF Materials Research Science and Engineering Center Program under awards DMR 08-20054 and DMR 14-20709. Use of the Center for Nanoscale Materials and Advanced Photon Source was supported by the DOE, Office of Science, Office of Basic Energy Sciences, under contract DE-AC02-06CH11357. MRCAT operations are supported by the DOE and MRCAT host institutions.


**SUPPORTING ONLINE MATERIAL**

www.sciencemag.org/content/347/6220/425/suppl/DC1
Materials and Methods
Figs. S1 to S16
Tables S1 to S3
References (*31*–*48*)

27 August 2014; accepted 8 December 2014
10.1126/science.1260501

DRUG RESISTANCE

# K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates

**Judith Straimer,[1] Nina F. Gnädig,[1] Benoit Witkowski,[2*] Chanaki Amaratunga,[3*] Valentine Duru,[2*] Arba Pramundita Ramadani,[4,5*†] Mélanie Dacheux,[1] Nimol Khim,[2] Lei Zhang,[6] Stephen Lam,[6] Philip D. Gregory,[6] Fyodor D. Urnov,[6] Odile Mercereau-Puijalon,[7] Françoise Benoit-Vical,[4,5‡] Rick M. Fairhurst,[3‡] Didier Ménard,[2‡] David A. Fidock[1,8§]**

**The emergence of artemisinin resistance in Southeast Asia imperils efforts to reduce the global malaria burden. We genetically modified the *Plasmodium falciparum K13* locus using zinc-finger nucleases and measured ring-stage survival rates after drug exposure in vitro; these rates correlate with parasite clearance half-lives in artemisinin-treated patients. With isolates from Cambodia, where resistance first emerged, survival rates decreased from 13 to 49% to 0.3 to 2.4% after the removal of K13 mutations. Conversely, survival rates in wild-type parasites increased from ≤0.6% to 2 to 29% after the insertion of K13 mutations. These mutations conferred elevated resistance to recent Cambodian isolates compared with that of reference lines, suggesting a contemporary contribution of additional genetic factors. Our data provide a conclusive rationale for worldwide K13-propeller sequencing to identify and eliminate artemisinin-resistant parasites.**

The worldwide use of artemisinin (ART)–based combination therapies (ACTs) for the treatment of *Plasmodium falciparum* malaria is the foundation of renewed efforts to eradicate this leading cause of childhood mortality (*1*, *2*). The pharmacodynamic properties of clinically used ART derivatives [artesunate, artemether, and dihydroartemisinin (DHA)] can reduce the biomass of drug-sensitive parasites by four orders of magnitude every 48 hours (*3*), corresponding to a single cycle of asexual blood-stage *P. falciparum* development. The short half-life (typically <1 hour) of ART derivatives in plasma necessitates the use of longer-lasting partner drugs that can eliminate residual parasites once the ART component has dropped to subtherapeutic concentrations (*4*). The use of ACTs in expanded malaria control and elimination programs has yielded notable successes in recent years, contributing to an estimated 30% reduction in global mortality rates in the past decade (*5*).

These impressive gains, however, are now threatened by the emergence of ART resistance, first detected in western Cambodia and now observed in Thailand, Vietnam, and Myanmar (*6*, *7*). The severity of this situation is underscored by the fact that resistance to piperaquine, an ACT partner drug, is emerging in western Cambodia (*8*, *9*). No alternative, fully effective first-line therapy is currently available to replace ACTs, should ART fail globally. Clinically, ART resistance is defined as a long parasite clearance half-life (the time it takes for the peripheral blood parasite density to decrease by 50%) after treatment with ART monotherapy or an ACT (*6*, *10*, *11*). This metric correlates with the percentage of early "ring-stage" parasites (0 to 3 hours after invasion of human erythrocytes) that survive a pharmacologically relevant exposure to DHA (the active metabolite of all ARTs), as measured in the in vitro Ring-stage Survival Assay ($RSA_{0\text{-}3h}$) (*12*).

Recently, mutations in the propeller domain of the *K13* gene were identified as candidate molecular markers of ART resistance (*13*). This gene resides on chromosome 13 of the *P. falciparum* genome, near regions earlier associated with slow parasite clearance rates (*14*–*16*). K13 belongs to the kelch superfamily of proteins, whose propeller domain harbors multiple protein-protein interaction sites and mediates diverse cellular functions, including ubiquitin-regulated protein degradation and oxidative stress responses (*17*). The K13 M476I mutation was first observed in Tanzanian F32 parasites that were exposed in vitro to escalating concentrations of ART over 5 years, yielding the F32-ART line (*13*, *18*). [Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. In the mutants, other amino acids were substituted at certain locations; for example, M476I indicates that methionine at position 476 was replaced by isoleucine.] Subsequent genomic analysis of Cambodian isolates identified four prevalent K13-propeller mutations (Y493H, R539T, I543T, and C580Y) that were associated with elevated $RSA_{0\text{-}3h}$ survival rates in vitro and long parasite clearance half-lives (>5 hours) in patients (*13*, *19*).

[1]Department of Microbiology and Immunology, Columbia University College of Physicians and Surgeons, New York, NY, USA. [2]Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia. [3]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. [4]Centre National de la Recherche Scientifique (CNRS), Laboratoire de Chimie de Coordination UPR8241, Toulouse, France. [5]Université de Toulouse, UPS, Institut National Polytechnique de Toulouse, Toulouse, France. [6]Sangamo BioSciences, Richmond, CA, USA. [7]Institut Pasteur, Parasite Molecular Immunology Unit, Paris, France. [8]Division of Infectious Diseases, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY, USA.
*These authors contributed equally to this work. †Present address: Department of Pharmacology and Therapy, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. ‡These authors contributed equally to this work. §To whom correspondence should be addressed. E-mail: df2260@columbia.edu

Determining whether K13-propeller mutations confer ART resistance in clinical isolates and assessing the contributions of individual polymorphisms in distinct genetic backgrounds is essential to defining the underlying molecular mechanisms.

We developed zinc-finger nucleases (ZFNs) (*20*) to enable targeted genetic engineering of *K13* in newly culture-adapted Cambodian isolates and older established reference lines of *P. falciparum* (tables S1 and S2). ZFNs were introduced into cultured intra-erythrocytic parasites via electroporation with plasmids containing *K13* donor templates. ZFNs triggered double-stranded breaks in the *K13* genomic target locus of this haploid organism, leading to DNA resection and repair events that captured mutations delivered by pZFN$^{K13}$-hdhfr plasmids (fig. S1). Donor plasmids contained additional synonymous mutations that preclude ZFN binding while preserving the K13-translated amino acid sequence across that same stretch of DNA base pairs. These silent ZFN binding-site mutations protected the donor sequence and prevented the edited recombinant locus from being recleaved by the nucleases. Plasmids contained either the wild-type *K13* allele or one of several mutations (present in the six-blade K13-propeller domain) found in ART-resistant Cambodian isolates or F32-ART. This strategy successfully introduced or removed mutations in a set of *P. falciparum* clinical isolates from Cambodia, the epicenter of emerging ART resistance, as well as reference laboratory lines from distinct geographic origins (Fig. 1 and table S3). Of note, $RSA_{0\text{-}3h}$ assays comparing parental and edited control parasites showed no difference if only the binding-site mutations were introduced into the K13-propeller domain, indicating that these synonymous mutations were phenotypically silent (Fig. 1 and fig. S2). Independent assays with the same parasite lines tested by our different groups yielded consistent survival rates between laboratories (fig. S3).

Using donor plasmids containing a wild-type *K13*-propeller sequence and silent binding-site mutations, we generated a series of clones in which individual K13 mutations were removed from ART-resistant Cambodian isolates. One of these isolates (Cam3.II) showed slow clearance after ART monotherapy (in vivo half-life 6.0 hours) (table S2). Parental Cam3.I$^{R539T}$ and Cam3.II$^{R539T}$ isolates harboring the R539T mutation showed 40 to 49% $RSA_{0\text{-}3h}$ survival, whereas edited Cam3.I$^{rev}$ and Cam3.II$^{rev}$ clones carrying the reverted wild-type allele showed only 0.3 to 0.7% survival (Fig. 2, A and B, and table S4). These highly significant differences in the survival rates of ring-stage parasites exposed to elevated DHA concentrations confirm the importance of R539T in mediating in vitro ART resistance in Cambodian isolates. Significant reductions in $RSA_{0\text{-}3h}$ survival rates were also observed upon removal of I543T (43% in Cam5$^{I543T}$ versus 0.3% in Cam5$^{rev}$) (Fig. 2C) and C580Y (13% in Cam2$^{C580Y}$ versus 2.4% in Cam2$^{rev}$) (Fig. 2D).

We also assessed the impact of introducing K13 mutations into a fast-clearing Cambodian isolate (CamWT; in vivo half-life 3.7 hours) (table S2), the Cam3.II$^{rev}$ clone, and three reference lines (V1/S, F32-TEM, and FCB). CamWT and Cam3.II$^{rev}$ parasites harboring wild-type *K13* alleles showed 0.6 to 0.7% $RSA_{0\text{-}3h}$ survival, whereas the corresponding C580Y-edited clones yielded 9 and 24% survival, respectively (Fig. 2, E and F). Introducing R539T into V1/S caused a similar increase in $RSA_{0\text{-}3h}$ survival (0.3 to 21%) (Fig. 2G and table S4). Editing F32-TEM to express M476I caused a moderate increase in $RSA_{0\text{-}3h}$ survival (<0.2% in F32-TEM to 1.7% in F32-TEM$^{M476I}$) (Fig. 2H). We also observed modest in vitro resistance in FCB parasites edited to express C580Y, with $RSA_{0\text{-}3h}$ survival increasing from 0.3% in the parental line to 1.9% in FCB$^{C580Y}$ parasites (Fig. 2I). This result differs from a recent study of the use of Cas9 in *P. falciparum*, which reported a greater increase in $RSA_{0\text{-}3h}$ survival (11 to 15%) in two clones engineered to express K13 C580Y (*21*). That report used the drug-sensitive NF54 strain—which was isolated decades before ART use and the emergence of resistance (*22*)—and did not examine additional mutations or assess the impact of removing K13 mutations from ART-resistant clinical isolates.

In contrast to the substantial changes we observed in the $RSA_{0\text{-}3h}$, standard in vitro dose-response measurements by use of parental and *K13*-edited V1/S and Cam3.II parasites revealed no effect of R539T or C580Y on DHA or artesunate median inhibitory concentration ($IC_{50}$) values (fig. S4). This finding is consistent with earlier studies that showed no correlation between $IC_{50}$ values and clinical ART resistance (*6*, *10*, *12*).

We subsequently investigated whether individual mutations confer different levels of ART resistance in the $RSA_{0\text{-}3h}$. In the Dd2 reference line, the introduction of M476I, R539T, or I543T mutations conferred considerably higher degrees of resistance than those of Y493H and C580Y (10 to 30% versus 2 to 4% survival, respectively) (Fig. 2J and table S4). These data corroborate the recent observation of higher levels of in vitro resistance in Cambodian isolates containing the R539T mutation as compared with Y493H or C580Y (*23*).

The relatively modest increase in survival of C580Y-expressing Dd2 parasites compared with

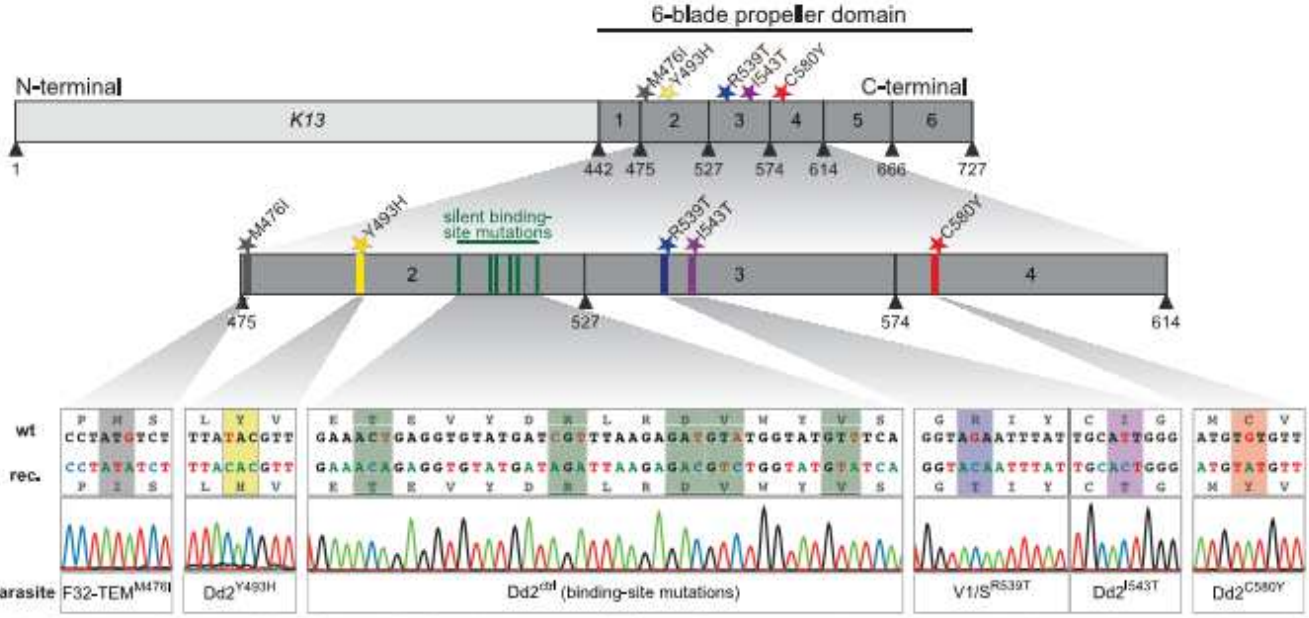



**Fig. 1. Genetic modification of the K13-propeller domain.** Location of K13-propeller mutations and sequencing results showing the insertion of individual mutations into recombinant parasites used in the $RSA_{0\text{-}3h}$. Dd2$^{ctrl}$ parasites contain only synonymous, phenotypically silent binding-site mutations and showed 0.7% survival rates, which is equivalent to those of parental Dd2 parasites (fig. S2).

R539T- and I543T-expressing clinical isolates and edited clones was quite unexpected, given that C580Y has rapidly become the predominant mutant allele in western Cambodia (*7*, *13*). We thus explored the impact of C580Y in different genetic backgrounds. Introducing C580Y conferred greater levels of resistance in three Cambodian isolates as compared with Dd2 and FCB parasites (Fig. 2K), suggesting a role for additional parasite factors in augmenting K13-mediated resistance in these contemporary field isolates. The disparity between relatively low in vitro resistance conferred by C580Y and its widespread dissemination in Cambodia might be explained by a lower fitness cost or increased transmission potential of C580Y-expressing parasites, or by the parasite genetic background.

Cambodian parasites are specifically characterized by sympatric subpopulations that show only limited genetic admixture and that generally harbor distinct K13 mutations (*16*). These findings suggest that K13 mutations might have arisen preferentially on backgrounds with favorable genetic factors. In this context, recent comprehensive analyses of K13 mutations across multiple sites in Southeast Asia have documented a series of additional mutations associated with slow clearance rates in Cambodia, Thailand, Myanmar, Laos, and Vietnam (*7*, *24*). K13 mutations have also been observed in African isolates (*7*, *25*, *26*), although none of these correspond to the most prevalent mutations in Cambodia, and ART or ACT treatments in African sites continue to show a high level of efficacy (*7*). A recent deep-sequencing study of the K13-propeller domain in more than 1110 *P. falciparum* infections collected from 14 sites across sub-Saharan Africa identified a large reservoir of naturally occurring K13-propeller variation, whose impact on artemisinin susceptibility is unknown and requires further investigation. These polymorphisms include one rare mutation previously observed in Cambodia (P553L) and several others (including A578S) close to known resistance-causing mutations in the propeller domain (*26*). Our gene-editing system can now be used to comprehensively dissect K13 polymorphisms across malaria-endemic regions and identify those that confer ring-stage ART resistance.

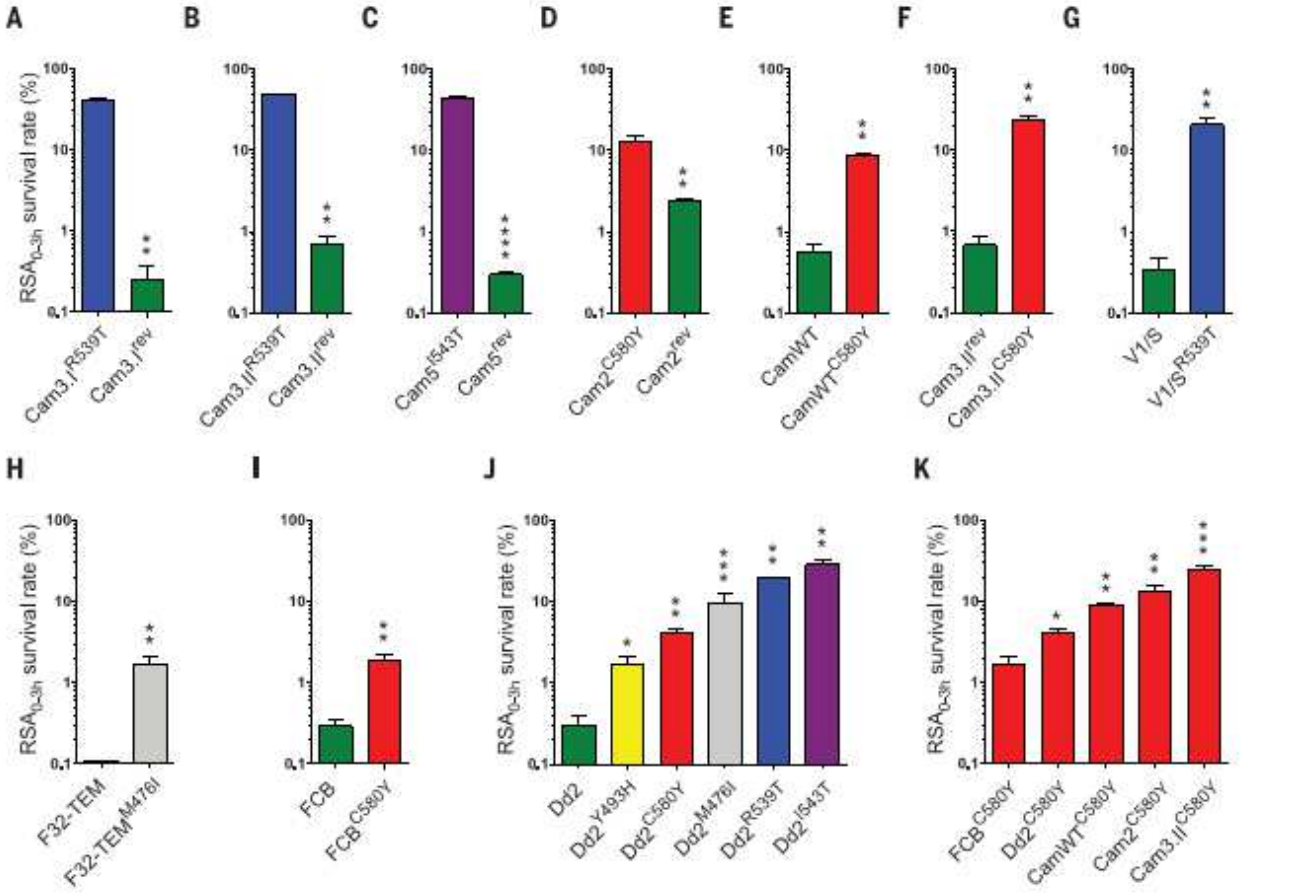



**Fig. 2. K13-propeller mutations confer artemisinin resistance in clinical isolates and reference lines in vitro, as defined in the $RSA_{0\text{-}3h}$.** Results show the percentage of early ring-stage parasites (0 to 3 hours after invasion of human erythrocytes) that survived a 6-hour pulse of 700 nM DHA (a pharmacologically relevant concentration of the active metabolite of ARTs), as measured by microscopy 66 hours later. Data show mean ± SEM percent survival compared with control dimethyl sulfoxide–treated parasites processed in parallel. (**A** to **D**) $RSA_{0\text{-}3h}$ survival for Cambodian isolates harboring native K13 mutations (shown in superscript) and ZFN-edited isogenic clones carrying wild-type *K13* alleles (superscript "rev"). (**E** to **I**) $RSA_{0\text{-}3h}$ survival for Cambodian isolates and reference lines harboring wild-type *K13* alleles and ZFN-edited isogenic clones carrying individual K13 mutations (shown in superscripts). (**J**) Impact of different K13 mutations on $RSA_{0\text{-}3h}$ survival in the Dd2 reference line, showing that I543T and R539T confer the highest levels of resistance. (**K**) Introduction of C580Y into multiple Cambodian clinical isolates and reference lines, showing that this mutation confers varying degrees of in vitro resistance depending on the parasite genetic background. The geographic origins and known drug-resistance genotypes of these isolates and lines are provided in table S2. Results were obtained from 3 or 4 independent assays performed in duplicate (values provided in table S4; F32-TEM showed <0.2% $RSA_{0\text{-}3h}$ survival). Two-sample *t* tests with unequal variances (performed with the STATA package) were used to assess for statistically significant differences between *K13*-edited clones and their comparator lines—the parental isolates listed on the left in (A) to (J) and the $FCB^{C580Y}$ clone in (K) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Statistical outputs (including calculations of the SE of the difference between the means of samples being compared and the *P* values) are listed in the supplementary materials.

Mode-of-action studies have shown that ARTs are active against all asexual blood stages of parasite development. In the more mature trophozoite stages, ARTs are activated after hemoglobin degradation and liberation of reactive heme whose iron moiety can cleave the endoperoxide linkage of these sesquiterpene lactone drugs (*27*). Activation generates free radicals that are thought to trigger oxidative stress and damage cellular macromolecules, including parasite membrane components, proteins, and neutral lipids (*28*, *29*). Recent evidence suggests that hemoglobin degradation begins early after merozoite invasion, potentially providing a source of ART activator in ring-stage parasites (*30*). Our $RSA_{0\text{-}3h}$ data support earlier evidence that reduced ring-stage susceptibility accounts for the clinical phenotype of slow parasite clearance after ART treatment (*12*, *31*). K13 mutations might achieve this by protecting parasites from the lethal effects of ART-induced oxidative damage, potentially via a cellular pathway similar to antioxidant transcriptional responses regulated by the mammalian ortholog Keap1 (*32*). Our set of K13-modified isogenic parasites with different levels of ART resistance on distinct genetic backgrounds now enables a search for K13-interacting partners and delivers tools to interrogate the underlying mechanism.

Our data demonstrate a central, causal role for K13-propeller mutations in conferring ART resistance in vitro and provide a molecular explanation for slow parasite clearance rates in patients (*6*, *7*, *10*). By exposing greater parasite biomasses to ACTs in vivo, K13-propeller mutations may promote the evolution of partner drug resistance (*8*, *9*) and higher-grade ART resistance. Our study thus offers a conclusive rationale for a global *K13* sequencing effort to track the spread of ART resistance and mitigate its impact on malaria treatment and control programs, particularly in hyperendemic regions in Africa.

## REFERENCES AND NOTES


1. R. Feachem, O. Sabot, *Lancet* 371, 1633–1635 (2008).
2. N. J. White *et al.*, *Lancet* 383, 723–735 (2014).
3. N. J. White, *Parasitology* 41, 301–308 (1999).
4. R. T. Eastman, D. A. Fidock, *Nat. Rev. Microbiol.* 7, 864–874 (2009).
5. World Health Organization, World Malaria Report: 2013. WHO Press: Geneva, Switzerland; available at www.who.int/malaria/publications/world_malaria_report_2013/en.
6. A. M. Dondorp *et al.*, *N. Engl. J. Med.* 361, 455–467 (2009).
7. E. A. Ashley *et al.*, *N. Engl. J. Med.* 371, 411–423 (2014).
8. D. L. Saunders *et al.*, *N. Engl. J. Med.* 371, 484–485 (2014).
9. R. Leang *et al.*, *Antimicrob. Agents Chemother.* 57, 818–826 (2013).
10. C. Amaratunga *et al.*, *Lancet Infect. Dis.* 12, 851–858 (2012).
11. J. A. Flegg *et al.*, *Malar. J.* 12, 411 (2013).
12. B. Witkowski *et al.*, *Lancet Infect. Dis.* 13, 1043–1049 (2013).
13. F. Ariey *et al.*, *Nature* 505, 50–55 (2014).
14. I. H. Cheeseman *et al.*, *Science* 336, 79–82 (2012).
15. S. Takala-Harrison *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 110, 240–245 (2013).
16. O. Miotto *et al.*, *Nat. Genet.* 45, 648–655 (2013).
17. J. Adams, R. Kelso, L. Cooley, *Trends Cell Biol.* 10, 17–24 (2000).
18. B. Witkowski *et al.*, *Antimicrob. Agents Chemother.* 54, 1872–1877 (2010).
19. C. Amaratunga, B. Witkowski, N. Khim, D. Menard, R. M. Fairhurst, *Lancet Infect. Dis.* 14, 449–450 (2014).
20. J. Straimer *et al.*, *Nat. Methods* 9, 993–998 (2012).
21. M. Ghorbal *et al.*, *Nat. Biotechnol.* 32, 819–821 (2014).
22. T. Ponnudurai, J. H. Meuwissen, A. D. Leeuwenberg, J. P. Verhave, A. H. Lensen, *Trans. R. Soc. Trop. Med. Hyg.* 76, 242–250 (1982).
23. C. Amaratunga *et al.*, *Antimicrob. Agents Chemother.* 58, 4935–4937 (2014).
24. S. Takala-Harrison *et al.*, *J. Infect. Dis.* 10.1093/infdis/jiu491 (2014).
25. M. D. Conrad *et al.*, *PLOS One* 9, e105690 (2014).
26. S. M. Taylor *et al.*, *J. Infect. Dis.* 10.1093/infdis/jiu467 (2014).
27. N. Kloris, D. J. Creek, L. Tilley, *Curr. Opin. Microbiol.* 16, 722–727 (2013).
28. C. L. Hartwig *et al.*, *Biochem. Pharmacol.* 77, 322–336 (2009).
29. T. Antoine *et al.*, *J. Antimicrob. Chemother.* 69, 1005–1016 (2014).
30. N. Kloris *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 108, 11405–11410 (2011).
31. S. Saralamba *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 108, 397–402 (2011).
32. Y. S. Keum, B. Y. Choi, *Molecules* 19, 10074–10089 (2014).



### ACKNOWLEDGMENTS

D.A.F. gratefully acknowledges funding from the NIH (R01 AI109023). This study was supported in part by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, NIH, the French "Agence Nationale de la Recherche" (ANR-13-BSV3-0018-01 and the Laboratoire d'Excellence IBEID), and the Institut Pasteur, Division International (ACIP A-10-2010). Parental and transgenic parasite lines have been deposited and are being made available through BEI Resources (www.mr4.org) with the following accession numbers: MRA-1240, Cam3.IR539T (also known as IPC 5202); MRA-1252, Cam3.Irev; MRA-1241, Cam6I543T (also known as IPC 4912); MRA-1253, Cam6rev; MRA-1236, Cam2C580Y (also known as IPC 3445); MRA-1254, Cam2rev; MRA-1250, CamWT; MRA-1251, CamWTC580Y; MRA-150, Dd2; MRA-1255, Dd2R539T. Parasite lines generated for this study will also be provided upon request from D.A.F. Requests for ZFNs should be directed to F.D.U. (FUrnov@sangamo.com); a materials transfer agreement is required. We extend our gratitude to F. Ariey (Institut Pasteur, Paris) for his important contribution to initiating this study, I. McKeague and O. Lieberman (Columbia University Medical Center) for their statistical and scientific input, and E. Rebar and the Production Group at Sangamo BioSciences for ZFN assembly and validation. L.Z., S.L., P.D.G., and F.D.U. declare that they are full-time employees of Sangamo, which designed, validated, and provided the ZFNs used in this study. B.W., Q.M.-P., F.B.-V., and D.M. are co-inventors on the pending patents US61/934651 and US62/062439, and N.K. is a co-inventor on the pending patent US62/062439. Both patents are filed by Institut Pasteur. These patents cover the use of K13 mutations as a molecular marker of *P. falciparum* ART resistance. Sangamo holds patents on engineered DNA-binding proteins and the use thereof in targeted genome engineering and gene-specific regulation. All other authors declare no competing financial interests.

### SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/347/6220/428/suppl/DC1
Materials and Methods
Figs. S1 to S4
Tables S1 to S5
Results of two-sample *t* tests with unequal variances
References (*33*–*36*)

5 September 2014; accepted 1 December 2014
Published online 11 December 2014;
10.1126/science.1260867


DRUG RESISTANCE

# Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance


**Sachel Mok,[1] Elizabeth A. Ashley,[2,3] Pedro E. Ferreira,[1] Lei Zhu,[1] Zhaoting Lin,[1] Tomas Yeo,[1] Kesinee Chotivanich,[4] Mallika Imwong,[5] Sasithon Pukrittayakamee,[4] Mehul Dhorda,[3,6,7] Chea Nguon,[8] Pharath Lim,[8,9] Chanaki Amaratunga,[9] Seila Suon,[8] Tran Tinh Hien,[10] Ye Htut,[11] M. Abul Faiz,[12] Marie A. Onyamboko,[13] Mayfong Mayxay,[14,15] Paul N. Newton,[2,3,14] Rupam Tripura,[2] Charles J. Woodrow,[2,3] Olivo Miotto,[2,16,17] Dominic P. Kwiatkowski,[16,17] François Nosten,[3,18] Nicholas P. J. Day,[2,3] Peter R. Preiser,[1] Nicholas J. White,[2,3] Arjen M. Dondorp,[2,3] Rick M. Fairhurst,[9] Zbynek Bozdech[1]***



Artemisinin resistance in *Plasmodium falciparum* threatens global efforts to control and eliminate malaria. Polymorphisms in the kelch domain–carrying protein K13 are associated with artemisinin resistance, but the underlying molecular mechanisms are unknown. We analyzed the in vivo transcriptomes of 1043 *P. falciparum* isolates from patients with acute malaria and found that artemisinin resistance is associated with increased expression of unfolded protein response (UPR) pathways involving the major PROSC and TRiC chaperone complexes. Artemisinin-resistant parasites also exhibit decelerated progression through the first part of the asexual intraerythrocytic development cycle. These findings suggest that artemisinin-resistant parasites remain in a state of decelerated development at the young ring stage, whereas their up-regulated UPR pathways mitigate protein damage caused by artemisinin. The expression profiles of UPR-related genes also associate with the geographical origin of parasite isolates, further suggesting their role in emerging artemisinin resistance in the Greater Mekong Subregion.


Artemisinin resistance in *Plasmodium falciparum* is spreading rapidly throughout Southeast Asia. Since it was first detected 7 years ago in Pailin, western Cambodia (*1*), artemisinin resistance has become prevalent in other Cambodian provinces (*2*, *3*), Thailand-Myanmar border areas (*4*, *5*), and southern Vietnam (*4*, *6*) and is emerging in southern Laos and central Myanmar (*4*). Artemisinin resistance threatens the

**D. Dihydroartemisinin-Piperaquine Resistance in Kupang**

Regarding the emergence of ART resistance and its spread in Asia, it is important to evaluate the level of ACTs resistance in Indonesia. Monitoring of K13 mutations is part of the WHO criteria to investigate ART resistance worldwide. Consequently, this gene is important to be examined during malaria clinical trials in Kupang to evaluate the possible ART resistance emergence in Indonesia.

Even though there was no report of any treatment failure yet after ACTs treatment and linked to ART resistance in Indonesia, this study was undertaken on isolates coming from areas where *P. falciparum* has been recorded as strongly present to assess whether ACTs resistance can be detected through *ex-vivo* RSA assay.

Ethics approval submission was the first step of the research parallel to a study of the population to define the most appropriate area among the endemic areas of malaria in Indonesia. Patients were eligible if they were aged 2-65 years, diagnosed for non-complicated malaria caused by particularly *P. falciparum* (slide-confirmed malaria), having fever (a tympanic temperature $\geq$ 37.5ºC) or history of fever 48 h preceding presentation and parasite density no more than 200,000 parasites per µL with minimum 0.5% parasitemia, seeking treatment in primary health center or regency referral hospital, and agreeing to sign the informed consent and follow the study. Patients were excluded if they were pregnant or lactating women, children under 5 kg, already taking malaria treatment for 4 weeks in advance, diagnosed for complicated malaria (co-infection), infected by other *Plasmodium* species than *P. falciparum*, and having signs of severe malaria, severe malnutrition, serious underlying disease (renal, cardiac, or hepatic), or known allergy to the study drugs.

Dihydroartemisinin-Piperaquine (Darplex®) was administered during the first 3 days of the study and a follow-up was conducted until day 42. To perform *ex-vivo* RSA and K13 gene

identification, parasites were isolated from 3 mL of patient's venous blood placed in ACD coagulant tube just prior to taking the drug. A couple drops of blood were examined for its parasitemia (thin smear), ± 100 µL was dropped into filter paper (Whatman® paper, Human ID Bloodstain card) for DNA extraction, and the remaining was prepared for RSA *ex-vivo*.


Figure 13. Position of Kupang (circled) in the map of Indonesia [208].

After several evaluations based on the number of patients, laboratory facilities, and other factors, the most relevant area to conduct the research was Kupang Regency, East Nusa Tenggara, one of the areas with high malaria incidence in Indonesia. Furthermore, according to the Department of Health of Kupang (internal epidemiology data, 2015), there were 64,953

malaria cases in East Nusa Tenggara province and 831 positive cases of *P. falciparum* malaria found in Kupang Regency during 2014, and it was noted that there were 50 *P. falciparum* malaria patients seeking treatment in primary health-care center of Kupang Regency from January to June 2015 (based on a direct observation at the primary health-care center of Kupang Regency). The distance between Kupang Regency and the laboratory of BPOM Kupang as the laboratory center for conducting RSA assay also contributed to the choice of this population area. Since the patients' blood withdrawal had limited time to be tested for its ACT resistance, the distance between the primary health-care center and laboratory became important. In the meantime, the ethics approval (ref. number: KE/FK/222/EC) from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Gadjah Mada University, and research permits from each primary health-care center in Kupang Regency, referral hospital and BPOM Kupang were obtained prior to the beginning of the study.

After one year of study, only 6 patients of *P. falciparum* malaria enrolled among whom one was developing severe malaria and one refused to continue the study after a week of follow-up. As a result, only four patients met the criteria and were followed up for the study. The small number of *P. falciparum* malaria patients that had been found was contrary to the previous data collected from direct observation at the primary health-care centers of Kupang Regency (2015). It might be so because there was a decrease in the malaria prevalence in this area. Based on the report of the Department of Health, there were 36,128 malaria cases reported in this province during 2015 [209]. This number indicated a significant decline in malaria prevalence from 13 API level in 2014 to 7 in 2015. Additionally, the national annual parasite also reduced by 0.99 to 0.85 in 2015 [4]. This finding was likely to represent the limited number of cases in Indonesia, particularly during our study in Kupang. Furthermore, this phenomenon occurs not only in Indonesia but also in other countries. A recent publication mentioned that malaria cases were declining by 54 % for last 3 years in Greater Mekong Sub-region (GMS) [210]. Several

factors that might influence the decrease number of malaria cases were climatic and non-climatic.

Climatic factors include temperature, rainfall, and relative humidity. The optimum temperature for parasite development is 27ºC. Temperature raise delays the parasite development in the gut of mosquito (more than 10 days). In addition, when the rain is delayed, the breed of mosquitos decreases. Moreover, lower relative humidity reduces the survival time of mosquito to transmit malaria. Based on the data from Metrological, Climatological, and Geophysical Agency (BMKG), the temperature in Kupang reached 31.07ºC with 65-90% humidity and 362 mm average rainfall. This condition was highly likely to affect malaria transmission.

Non-climatic factors including urbanization and human host can have an impact on the transmission. The incidence of malaria is lower in urban area than in rural area, but the development in rural area may also influence malaria distribution. Moreover, there is a possibility that people already have immunity which makes better tolerance to the effects of malaria, and non-immune people believe that being infected by malaria is the same as common cold and does not need serious treatment in healthcare center. This makes many malaria cases under reported.

In addition, malaria elimination program run by the government also takes part in case diminution. Specific strategies have been implemented by the government to expedite the elimination program. Since Kupang is one of the high malaria endemic areas, the acceleration strategies have been employed through mass ITNs campaign, early malaria detection and appropriate treatment [4]. The evidence of the number of malaria cases reduction may indicate that the elimination program runs well.

From the six eligible patients, bloods were taken and prepared for *ex-vivo* RSA. Thin blood smears were made to confirm the diagnosis and determine the parasitemia ratio. Unfortunately, all the blood samples which were delivered to the laboratory could not allow the detection of parasite presence although it has been previously held as positive *P. falciparum* malaria. We did not have access to the original smears in order to re-confirm whether the initial parasitemia met the eligible criteria (> 0.5%). Therefore, the *ex-vivo* RSA could not be carried out though the analysis of K13 mutation is currently in progress. Some possibilities are offered to assess the causes and provide the solutions.

Theoretically, the blood must be processed right after having been taken since delayed processing can potentially undermine the parasite condition [211]. If the distance between the site of blood withdrawal and the laboratory to conduct the RSA assay is quite long, the blood should be transported in a cold temperature. It took approximately two hours from Kupang Regency to BPOM Kupang in Kupang city, therefore the patient's blood was stored in an ice box during transportation. However, this did not work. A sudden temperature change from the patient's body to the storage condition might contribute to this problem. Then, an approach was made by modifying the storage method. The blood was stored in a cold temperature (2-10ºC) during the transport to the laboratory and placed in a room temperature during the preparation in laboratory. Yet, it did not work either. Moreover, since the authorized working hours in the laboratory were limited, the blood preparation was not possible to process on the same day.

Another problem was related to the diagnosis process. The diagnosis was made by physicians based on patient's blood smears prepared by technicians. Not all of the technicians were certified giving an impact on smear evaluation. There was a possibility that false positive diagnosis occurred.

These findings lead us to the following perspectives. Looking for another feasible study area which has a large number of *P. falciparum* cases, adequate laboratory facility and more flexible access to conduct the study outside working hours are strongly suggested. Furthermore, in order to perform RSA assay, it is important to understand the Indonesian parasite characteristic by modification of blood transport and storage conditions if a field laboratory is not available. Also, as K13 has a great impact on malaria control in Cambodia, it is interesting to work on K13 screening not only in one endemic district but also in several other endemic regions in Indonesia to get whole a map of K13 existence in Indonesia.

# CONCLUSION AND PERSPECTIVES

## A. CONCLUSION

Various efforts and studies have been undertaken to reduce malaria prevalence all around the world. This study points out several strategies for combating malaria particularly in antimalarial drug resistance. The approaches to solve antimalarial resistance problem include discovering new drugs, understanding the resistance mechanism, and investigating the occurrence of ART resistance in endemic area through identification of K13 gene mutation.

The first approach is discovering new drug by assessing selected medicinal plants in Indonesia, namely *T. diversifolia, C. barbata, T. crispa, A. flava* and *P. cauliflora*. Those plants represent not only good antiplasmodial activity but also potential antibabesial and antileishmanial with *P. cauliflora* as the most promising medicinal plant for further investigation.

Potential new drugs are also assessed by synthesizing organometallic compounds. Gold complex combined with NHCs is prospective as a new antimalarial synthetic drug since it exerts good antiplasmodial activity against CQ-resistant strain, especially in the cationic form, and has lipophilic properties.

The second approach is done by understanding the role of K13-propeller mutation in artemisinin resistance. K13-propeller mutation mediates the occurrence of artemisinin resistance *in vitro* on both Cambodian clinical isolates and laboratory lines.

The last approach is identification of K13-gene mutation in Kupang, one of the endemic regions in Indonesia. Unfortunately, it could not be established due to the limited number of samples.

## B. PERSPECTIVES

As Indonesia government intends to achieve malaria-free state by 2030, several schemes on antimalarial drug resistance should be dealt with in advance. Some of the schemes were explored in this study resulting in several outlooks as follows.

First, selected plants from Indonesia have been proved to possess good antiplasmodial activity. Therefore, further phytochemical investigation is essential to explore the active compound of promising plants in order to discover new antimalarial drug. Additionally, a study on their mode of action will be more beneficial for the drug development.

Another drug source, chemical synthesis from gold compound combined with NHC as a ligand, also presents a good activity against CQ-resistant parasite. Hence, immediate investigation on advanced pharmacology assays is required, including exploration of pharmacokinetics profile, stability study, and toxicity study before continuing to a clinical level.

Third, exploring ART resistance mechanism has resulted in confirmation of K13-propeller mutation role as a mediator in ART resistance. Indeed, global attempts for K13 gene sequencing are worth making to figure out the distribution in Southeast Asian countries, particularly in several endemic areas in Indonesia. Moreover, K13 mutation monitoring is included in the WHO criteria to follow ART resistance worldwide.

Following the third perspective, the study on K13-propeller mutation in Kupang, one of the malaria endemic areas in Indonesia, has yet to be clearly conclusive. Follow-up attempts to investigate its occurrence are strongly suggested to look for another feasible study area which has a large number of *P. falciparum* cases, adequate laboratory facility and more flexible access to conduct the study outside working hours. Still, in order to perform RSA assay, it is

important to understand the Indonesian parasite characteristics by modification of blood transport and storage conditions if a field laboratory is not available. Also, as K13 has a great impact on malaria control in Cambodia, it is interesting to work on K13 screening not only in one endemic district but also in several other regions in Indonesia to obtain the whole map of K13 existence in Indonesia.

# REFERENCES


1. WHO, *World Malaria Report 2016*. 2016, WHO: Switzerland.
2. MoH, *Indonesia Malaria Report 2014*. 2014, Ministry of Health Republic of Indonesia: Jakarta.
3. Elyazar, I.R., S.I. Hay, and J.K. Baird, *Malaria distribution, prevalence, drug resistance and control in Indonesia.* Adv Parasitol, 2011. **74**: p. 41-175.
4. MoH, *Infodatin Malaria*. 2016, Ministry of Health of Indonesia.
5. WHO, *World Malaria Report 2015.* 2015.
6. DoH, *Profil Pengendalian Penyakit dan Penyehatan Lingkungan 2008*. 2009, Department of Health of Indonesia. p. 264.
7. Karou, D., et al., *African Ethnopharmacology and New Drug Discovery.* Med Aromat Plant Sci Biotechnol, 2007. **1**(1).
8. Saxena, S., et al., *Antimalarial agents from plants sources.* Curr Sci, 2003. **85**(9): p. 16.
9. Pinna, R.A., et al., *Malaria-Cutaneous Leishmaniasis Co-infection: Influence on Disease Outcomes and Immune Response.* Front Microbiol, 2016. **7**: p. 982.
10. Newman, D.J., G.M. Cragg, and K.M. Snader, *Natural products as sources of new drugs over the period 1981-2002.* J Nat Prod, 2003. **66**(7): p. 1022-37.
11. Hemmert, C., et al., *Antiplasmodial activities of gold(I) complexes involving functionalized N-heterocyclic carbenes.* Bioorg Med Chem, 2016. **24**(13): p. 3075-82.
12. DoH, *Pedoman penatalaksanaan pengobatan malaria*, D.K.R. Indonesia, Editor. 2008: Jakarta.
13. Asih, P.B., et al., *Seasonal distribution of anti-malarial drug resistance alleles on the island of Sumba, Indonesia.* Malar J, 2009. **8**: p. 222.
14. Rahman, M., et al. *Antimalarial drug resistance*. in *Regional Health Forum*. 2011.
15. WHO, *Guidelines For The treatment of Malaria.* 2006.
16. ter Kuile, F., et al., *Plasmodium falciparum: in vitro studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria.* Exp Parasitol, 1993. **76**(1): p. 85-95.
17. White, N.J., *Qinghaosu (artemisinin): the price of success.* Science, 2008. **320**(5874): p. 330-4.
18. Noedl, H., et al., *Evidence of artemisinin-resistant malaria in western Cambodia.* N Engl J Med, 2008. **359**(24): p. 2619-20.
19. Dondorp, A.M., et al., *Artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2009. **361**(5): p. 455-67.
20. Noedl, H., D. Socheat, and W. Satimai, *Artemisinin-resistant malaria in Asia.* N Engl J Med, 2009. **361**(5): p. 540-1.
21. Amaratunga, C., et al., *Dihydroartemisinin-piperaquine resistance in Plasmodium falciparum malaria in Cambodia: a multisite prospective cohort study.* Lancet Infect Dis, 2016.
22. Witkowski, B., et al., *Increased tolerance to artemisinin in Plasmodium falciparum is mediated by a quiescence mechanism.* Antimicrob Agents Chemother, 2010. **54**(5): p. 1872-7.
23. Witkowski, B., et al., *Reduced artemisinin susceptibility of Plasmodium falciparum ring stages in western Cambodia.* Antimicrob Agents Chemother, 2013. **57**(2): p. 914-923.
24. Ariey, F., et al., *A molecular marker of artemisinin resistant Plasmodium falciparum malaria.* nature, 2014: p. 1-6.
25. Straimer, J., et al., *Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates.* Science, 2015. **347**(6220): p. 428-31.
26. Ashley, E.A., et al., *Spread of artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2014. **371**(5): p. 411-23.
27. Takala-Harrison, S., et al., *Independent emergence of artemisinin resistance mutations among Plasmodium falciparum in Southeast Asia.* J Infect Dis, 2015. **211**(5): p. 670-9.
28. Menard, D., et al., *A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphisms.* N Engl J Med, 2016. **374**(25): p. 2453-64.
29. Ye, R., et al., *Distinctive origin of artemisinin-resistant Plasmodium falciparum on the China-Myanmar border.* Sci Rep, 2016. **6**: p. 20100.

30. Wang, Z., et al., *Prevalence of K13-propeller polymorphisms in Plasmodium falciparum from China-Myanmar border in 2007-2012.* Malar J, 2015. **14**: p. 168.
31. Wang, Z., et al., *Artemisinin resistance at the China-Myanmar border and association with mutations in the K13 propeller gene.* Antimicrob Agents Chemother, 2015. **59**(11): p. 6952-9.
32. Huang, F., et al., *A Single Mutation in K13 Predominates in Southern China and Is Associated With Delayed Clearance of Plasmodium falciparum Following Artemisinin Treatment.* J Infect Dis, 2015. **212**(10): p. 1629-35.
33. Meshnick, S.R. and M.J. Dobson, *The history of Antimalarial Drugs*, in *Antimalarial Chemotherapy: Mechanisms of Action, Resistance, and New Direction in Drug Discovery*, P.J. Rosenthal, Editor. 2001, Humana Press Inc: New Jersey.
34. Cox, F.E., *History of the discovery of the malaria parasites and their vectors.* Parasit Vectors, 2010. **3**(1): p. 5.
35. Anandan, J.V., *Parasitic disease*, in *Pharmacotherapy Principle and Practise*, M.A. Burns, Editor. 2008, Mc Graw Hill: New York. p. 1186-1189.
36. Berens-Riha, N., et al., *Plasmodium knowlesi found in several samples from Indonesia.* Internal Society for Infectious Disease, 2009.
37. Cox-Singh, J. and B. Singh, *Knowlesi malaria: newly emergent and of public health importance?* Trends Parasitol, 2008. **24**(9): p. 406-10.
38. Haldar, K., et al., *Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease.* Annu Rev Pathol, 2007. **2**: p. 217-49.
39. Fujioka, H. and M. Aikawa, *Structure and life cycle.* Chem Immunol, 2002. **80**: p. 1-26.
40. Lobo, C.A. and N. Kumar, *Sexual differentiation and development in the malaria parasite.* Parasitol Today, 1998. **14**(4): p. 146-50.
41. Sinden, R.E., *Gametocytogenesis of Plasmodium falciparum in vitro: an electron microscopic study.* Parasitology, 1982. **84**(1): p. 1-11.
42. Bousema, T., et al., *Asymptomatic malaria infections: detectability, transmissibility and public health relevance.* Nat Rev Microbiol, 2014. **12**(12): p. 833-40.
43. Fitch, C.D., *Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs.* Life Sci, 2004. **74**(16): p. 1957-72.
44. Greenwood, B.M., et al., *Malaria: progress, perils, and prospects for eradication.* J Clin Invest, 2008. **118**(4): p. 1266-76.
45. Wein, S., et al., *Transport and pharmacodynamics of albitiazolium, an antimalarial drug candidate.* Br J Pharmacol, 2012. **166**(8): p. 2263-76.
46. Robert, A., et al., *The antimalarial drug artemisinin alkylates heme in infected mice.* Proc Natl Acad Sci U S A, 2005. **102**(38): p. 13676-80.
47. O'Neill PM, et al., *4-Aminoquinolines-past, present, and futur : a chemical perspective.* Pharmacol. ther., 1998. **77-1**: p. 29-58.
48. Bloland, P.B., *Drug Resistance in Malaria*, WHO, Editor. 2001: USA.
49. Biamonte, M.A., J. Wanner, and K.G. Le Roch, *Recent advances in malaria drug discovery.* Bioorg Med Chem Lett, 2013. **23**(10): p. 2829-43.
50. Winstanley, P., *Modern chemotherapeutic options for malaria.* Lancet Infect Dis, 2001. **1**(4): p. 242-50.
51. Kremsner, P.G., et al., *Clindamycin treatment of falciparum malaria in Brazil.* J Antimicrob Chemother, 1989. **23**(2): p. 275-81.
52. Kremsner, P.G., et al., *Clindamycin in combination with chloroquine or quinine is an effective therapy for uncomplicated Plasmodium falciparum malaria in children from Gabon.* J Infect Dis, 1994. **169**(2): p. 467-70.
53. Lell, B. and P.G. Kremsner, *Clindamycin as an antimalarial drug: review of clinical trials.* Antimicrob Agents Chemother, 2002. **46**(8): p. 2315-20.
54. Losert, H., et al., *Experiences with severe P. falciparum malaria in the intensive care unit.* Intensive Care Med, 2000. **26**(2): p. 195-201.

55. Kremsner, P.G., et al., *Comparison of micronized halofantrine with chloroquine-antibiotic combinations for treating Plasmodium falciparum malaria in adults from Gabon.* Am J Trop Med Hyg, 1994. **50**(6): p. 790-5.
56. Metzger, W., et al., *High efficacy of short-term quinine-antibiotic combinations for treating adult malaria patients in an area in which malaria is hyperendemic.* Antimicrob Agents Chemother, 1995. **39**(1): p. 245-6.
57. Tu, Y., *The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine.* Nat Med, 2011. **17**(10): p. 1217-20.
58. Barradell, L.B. and A. Fitton, *Artesunate. A review of its pharmacology and therapeutic efficacy in the treatment of malaria.* Drugs, 1995. **50**(4): p. 714-41.
59. Golenser, J., et al., *Current perspectives on the mechanism of action of artemisinins.* Int J Parasitol, 2006. **36**(14): p. 1427-41.
60. de Ridder, S., F. van der Kooy, and R. Verpoorte, *Artemisia annua as a self-reliant treatment for malaria in developing countries.* Journal of Ethnopharmacology, 2008. **120**(3): p. 302-314.
61. Balint, G.A., *Artemisinin and its derivatives: an important new class of antimalarial agents.* Pharmacol Ther, 2001. **90**(2-3): p. 261-5.
62. Burk, O., et al., *Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor.* Mol Pharmacol, 2005. **67**(6): p. 1954-65.
63. Meshnick, S.R., *Artemisinin: mechanisms of action, resistance and toxicity.* Int J Parasitol, 2002. **32**(13): p. 1655-60.
64. Looareesuwan, S., et al., *Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand.* Am J Trop Med Hyg, 1996. **54**(1): p. 62-6.
65. Radloff, P.D., et al., *Atovaquone plus proguanil is an effective treatment for Plasmodium ovale and P. malariae malaria.* Trans. Roy. Soc. Trop. Med. Hyg., 1996. **90**: p. 682.
66. Srivastava, I.K. and A.B. Vaidya, *A mechanism for the synergistic antimalarial action of atovaquone and proguanil.* Antimicrob Agents Chemother, 1999. **43**(6): p. 1334-9.
67. Ringwald P., et al., *Randomised trial of pyronaridine versus chloroquine for acute uncomplicated falciparum malaria in Africa.* lancet, 1996. **347**: p. 24-27.
68. Looareesuwan, S., et al., *Pyronaridine.* Lancet, 1996. **347**(9009): p. 1189-90.
69. Olliaro, P.L. and P.L. Trigg, *Status of antimalarial drugs under development.* Bull. WHO, 1995. **73-5**: p. 565-571.
70. Vugt, M.V., et al., *Efficacy of six doses of artemether-lumefantrine (benflumetol) in multidrug-resistant Plasmodium falciparum malaria.* Am J Trop Med Hyg, 1999. **60**(6): p. 936-42.
71. German, P.I. and F.T. Aweeka, *Clinical pharmacology of artemisinin-based combination therapies.* Clin Pharmacokinet, 2008. **47**(2): p. 91-102.
72. Kokwaro, G., L. Mwai, and A. Nzila, *Artemether/lumefantrine in the treatment of uncomplicated falciparum malaria.* Expert Opin Pharmacother, 2007. **8**(1): p. 75-94.
73. Travassos, M.A. and M.K. Laufer, *Resistance to antimalarial drugs: molecular, pharmacologic, and clinical considerations.* Pediatr Res, 2009. **65**(5 Pt 2): p. 64R-70R.
74. White, N.J., M. van Vugt, and F. Ezzet, *Clinical pharmacokinetics and pharmacodynamics and pharmacodynamics of artemether-lumefantrine.* Clin Pharmacokinet, 1999. **37**(2): p. 105-25.
75. Byakika-Kibwika, P., et al., *Artemether-Lumefantrine Combination Therapy for Treatment of Uncomplicated Malaria: The Potential for Complex Interactions with Antiretroviral Drugs in HIV-Infected Individuals.* Malar Res Treat, 2011. **2011**: p. 703730.
76. McIntosh, H.M. and B.M. Greenwood, *Chloroquine or amodiaquine combined with sulfadoxine-pyrimethamine as a treatment for uncomplicated malaria--a systematic review.* Ann Trop Med Parasitol, 1998. **92**(3): p. 265-70.
77. WHO, *World Malaria Report 2014.* 2014.

78. Ubben, D. and E.M. Poll, *MMV in partnership: the Eurartesim(R) experience.* Malar J, 2013. **12**: p. 211.
79. WHO, *Status report on artemisinin and ACT resistance*. 2015, World Health Organization: Geneva.
80. Paloque, L., et al., *Plasmodium falciparum: multifaceted resistance to artemisinins.* Malar J, 2016. **15**: p. 149.
81. Asih, P.B., et al., *Efficacy of artemisinin-based combination therapy for treatment of persons with uncomplicated Plasmodium falciparum malaria in West Sumba District, East Nusa Tenggara Province, Indonesia, and genotypic profiles of the parasite.* Am J Trop Med Hyg, 2009. **80**(6): p. 914-8.
82. Harijanto, P.N., *Malaria treatment by using artemisinin in Indonesia.* Acta Med Indones, 2010. **42**(1): p. 51-6.
83. Sutanto, I., et al., *Good efficacy of artemether-lumefantrine for uncomplicated falciparum malaria in eastern Sumba, East Nusatenggara, Indonesia.* Acta Med Indones, 2012. **44**(3): p. 187-92.
84. Sutanto, I., et al., *Randomized, open-label trial of primaquine against vivax malaria relapse in Indonesia.* Antimicrob Agents Chemother, 2013. **57**(3): p. 1128-35.
85. Tjitra, E., et al., *Efficacy and safety of artemisinin-naphthoquine versus dihydroartemisinin-piperaquine in adult patients with uncomplicated malaria: a multi-centre study in Indonesia.* Malar J, 2012. **11**: p. 153.
86. MoH, *Permenkes RI No. 5 tahun 2013 tentang Pedoman Tata Laksana Malaria*, D.J.P.p.d.P. Lingkungan, Editor. 2015, Ministry of Health Republic of Indonesia: Jakarta.
87. Bloland, P.B., *Drug resistance in malaria*. WHO ed, ed. WHO/CDS/DRS. Vol. 2001.4. 2001, Geneva: WHO. 27.
88. Wongsrichanalai, C., et al., *Epidemiology of drug-resistant malaria.* Lancet Infect Dis, 2002. **2**(4): p. 209-18.
89. Phillips, R.S., *Current Status of Malaria and Potential for Control.* Clin Microbiol Rev, 2001. **14**(1): p. 208-226.
90. Echeverry, D.F., et al., *Polymorphisms in the pfcrt and pfmdr1 Genes of Plasmodium falciparum and in Vitro Susceptibility to Amodiaquine and Desethylamodiaquine*. 2007. p. 1034-1038.
91. Peel, S.A., et al., *A strong association between mefloquine and halofantrine resistance and amplification, overexpression, and mutation in the P-glycoprotein gene homolog (pfmdr) of Plasmodium falciparum in vitro.* Am J Trop Med Hyg, 1994. **51**(5): p. 648-58.
92. Pickard, A.L., et al., *Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1.* Antimicrob Agents Chemother, 2003. **47**(8): p. 2418-23.
93. Sidhu, A.B., et al., *Decreasing pfmdr1 copy number in plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin.* J Infect Dis, 2006. **194**(4): p. 528-35.
94. Sidhu, A.B., D. Verdier-Pinard, and D.A. Fidock, *Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations.* Science, 2002. **298**(5591): p. 210-3.
95. Fidock, D.A., et al., *Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance.* Mol Cell, 2000. **6**(4): p. 861-71.
96. Price, R.N., et al., *Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number.* Lancet, 2004. **364**(9432): p. 438-47.
97. Price, R.N., et al., *The pfmdr1 gene is associated with a multidrug-resistant phenotype in Plasmodium falciparum from the western border of Thailand.* Antimicrob Agents Chemother, 1999. **43**(12): p. 2943-9.
98. Peterson, D.S., W.K. Milhous, and T.E. Wellems, *Molecular basis of differential resistance to cycloguanil and pyrimethamine in Plasmodium falciparum malaria.* Proc. Natl. Acad. Sci. USA, 1990. **87**(8): p. 3018-22.

99. Wang, P., et al., *Sulfadoxine resistance in the human malaria parasite Plasmodium falciparum is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization.* Mol Microbiol, 1997. **23**(5): p. 979-86.
100. Sanchez, C.P., et al., *Transporters as mediators of drug resistance in Plasmodium falciparum.* Int J Parasitol, 2010. **40**(10): p. 1109-18.
101. Koenderink, J.B., et al., *The ABCs of multidrug resistance in malaria.* Trends Parasitol, 2010.
102. Venkatesan, M., et al., *Polymorphisms in Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes: parasite risk factors that affect treatment outcomes for P. falciparum malaria after artemether-lumefantrine and artesunate-amodiaquine.* Am J Trop Med Hyg, 2014. **91**(4): p. 833-43.
103. Kessl, J.J., S.R. Meshnick, and B.L. Trumpower, *Modeling the molecular basis of atovaquone resistance in parasites and pathogenic fungi.* Trends Parasitol, 2007. **23**(10): p. 494-501.
104. Coteron, J.M., et al., *Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies potent Plasmodium falciparum dihydroorotate dehydrogenase inhibitors with clinical candidate potential.* J Med Chem, 2011. **54**(15): p. 5540-61.
105. Ferone, R., *Folate metabolism in malaria.* Bull World Health Organ, 1977. **55**(2-3): p. 291-8.
106. Shaio, M. and P. Lee, *Molecular Mechanisms of Resistance of Plasmodium falciparum to Antifolates.* Kuang Tien Med J, 2007. **2**(4).
107. Ibraheem, Z.O., et al., *Role of Different Pfcrt and Pfmdr-1 Mutations in Conferring Resistance to Antimalaria Drugs in Plasmodium falciparum.* Malar Res Treat, 2014. **2014**: p. 950424.
108. Summers, R.L., M.N. Nash, and R.E. Martin, *Know your enemy: understanding the role of PfCRT in drug resistance could lead to new antimalarial tactics.* Cell Mol Life Sci, 2012. **69**(12): p. 1967-95.
109. Setthaudom, C., et al., *Role of Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes on in vitro chloroquine resistance in isolates of Plasmodium falciparum from Thailand.* Am J Trop Med Hyg, 2011. **85**(4): p. 606-11.
110. Gregson, A. and C.V. Plowe, *Mechanisms of resistance of malaria parasites to antifolates.* Pharmacol Rev, 2005. **57**(1): p. 117-45.
111. WHO, *Artemisinin and artemisinin-based combination therapy resistance*, in *Global Malaria Program*. 2016, WHO.
112. WHO, *Artemisinin and artemisinin-based combination therapy resistance*. 2016, World Health Organization: Geneva.
113. Nakaso, K., et al., *PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells.* FEBS Lett, 2003. **546**(2-3): p. 181-4.
114. Mbengue, A., et al., *A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria.* Nature, 2015. **520**(7549): p. 683-7.
115. Chen, N., et al., *Fatty acid synthesis and pyruvate metabolism pathways remain active in dihydroartemisinin-induced dormant ring stages of Plasmodium falciparum.* Antimicrob Agents Chemother, 2014. **58**(8): p. 4773-81.
116. Oask, S.C., et al., *Malria: Obstacle and Opportunities*. 1991, Washington: National Academy Press.
117. Olliaro, P., J. Cattani, and D. Wirth, *Malaria, the submerged disease.* JAMA, 1996. **275**(3): p. 230-3.
118. Alonso, P.L., et al., *The effect of insecticide-treated bed nets on mortality of Gambian children.* Lancet, 1991. **337**(8756): p. 1499-502.
119. Rosenthal, P.J., *Proteases of malaria parasites: new targets for chemotherapy.* Emerg Infect Dis, 1998. **4**(1): p. 49-57.
120. Rosenthal, P.J., *Antimalarial drug discovery: old and new approaches.* J Exp Biol, 2003. **206**(Pt 21): p. 3735-44.
121. Clarkson, C., et al., *In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa.* J Ethnopharmacol, 2004. **92**(2-3): p. 177-91.

122. Menan, H., et al., *Antiplasmodial activity and cytotoxicity of plants used in West African traditional medicine for the treatment of malaria.* J Ethnopharmacol, 2006. **105**(1-2): p. 131-6.
123. Mbatchi, S.F., et al., *In vitro antiplasmodial activity of 18 plants used in Congo Brazzaville traditional medicine.* J Ethnopharmacol, 2006. **104**(1-2): p. 168-174.
124. Phillipson, J.D., *Phytochemistry and medicinal plants.* Phytochemistry, 2001. **56**(3): p. 237-43.
125. Saxena, S., et al., *Antimalarial agents from plant sources.* Current science, 2003. **85**(9): p. 1314-1329.
126. Klayman, D.L., et al., *Isolation of artemisinin (qinghaosu) from Artemisia annua growing in the United States.* J Nat Prod, 1984. **47**(4): p. 715-7.
127. Teixeira da Silva, J.A., *Mining the essenthial oils of the Anthemideae.* African J Biotech, 2004. **3**(12).
128. Warhurst, D.C., *New drugs and their potential use against drug-resistant malaria.* Ann Ist Super Sanita, 1985. **21**(3): p. 327-36.
129. Anonymous, *Herbal Pharmacology in the People Republic of China*, N.A.o. Science, Editor. 1975: Washington.
130. Steck, E.A., *The Chemotherapy of Protozoan Disease.* Washington Ed. ed. *Walter Reed Army Institute of Research*, ed. *W.R.A.I.o. Research*. 1972.
131. Mojab, F., *Antimalarial natural products: a review.* Avicenna J Phytomed, 2012. **2**(2): p. 52-62.
132. Basco, L.K., et al., *In vitro activities of Furoquinoline and Acridone alkaloids against Plasmodium falciparum.* Antimicrob. Agents Chemother., 1994. **38-5**: p. 1169-1171.
133. Dolabela, M.F., et al., *In vitro antiplasmodial activity of extract and constituents from Esenbeckia febrifuga, a plant traditionally used to treat malaria in the Brazilian Amazon.* Phytomedicine, 2008. **15**(5): p. 367-72.
134. Muriithi, M.W., et al., *Isolation and in vitro antiplasmodial activities of alkaloids from Teclea trichocarpa: in vivo antimalarial activity and X-ray crystal structure of normelicopicine.* J Nat Prod, 2002. **65**(7): p. 956-9.
135. Willcox, M., et al., *Do ethnobotanical and laboratory data predict clinical safety and efficacy of anti-malarial plants?* Malar J, 2011. **10 Suppl 1**: p. S7.
136. USDA, *Plant database*. 2017, Natural Resources Conservation Services: United States.
137. Njoroge, G.N. and R.W. Bussmann, *Diversity and utilization of antimalarial ethnophytotherapeutic remedies among the Kikuyus (Central Kenya).* J Ethnobiol Ethnomed, 2006. **2**: p. 8.
138. Kandungu, J., et al., *Pesticidal plant leaflet: Tithonia diversifolia (Hemsley) A. Gray*, U.o. Greenwich, Editor. 2013: United Kingdom.
139. Oyewole, I.O., et al., *Anti-malarial and repellant activities of Thitonia diversifolia (Hemsl.) leaf extracts.* J Med Plants Res, 2008. **2**(8): p. 5.
140. Goffin, E., et al., *In vitro antiplasmodial activity of Tithonia diversifolia and identification of its main active constituent: tagitinin C.* Planta Med, 2002. **68**(6): p. 543-5.
141. Elufioye, T.O. and J.M. Agbedahunsi, *Antimalarial activities of Tithonia diversifolia (Asteraceae) and Crossopteryx febrifuga (Rubiaceae) on mice in vivo.* J Ethnopharmacol, 2004. **93**(2-3): p. 167-71.
142. Manilal, K.S. and T. Sabu, *Cyclea barbata miers (menispermaceae): a new record of a medicinal plant from South India.* Anc Sci Life, 1985. **4**(4): p. 229-31.
143. Siregar, I.M. and I. Miladiyah, *Protective effects of Cyclea barbata Miers leaves against aspirin-induced gastric ulcer in mice.* Univ Med, 2011. **30**(2).
144. Lin L.Z., et al., *Cytotoxicc and antimalarial bisbenzylisoquinoline alkaloids from Cyclea barbata.* Journal of natural products, 1993. **56-1**: p. 22-29.
145. Guinaudeau, H., et al., *Bisbenzylisoquinoline alkaloids from Cyclea barbata.* J Nat Prod, 1993. **56**(11): p. 1989-92.

146. Ahmad, W., I. Jantan, and S.N. Bukhari, *Tinospora crispa (L.) Hook. f. & Thomson: A Review of Its Ethnobotanical, Phytochemical, and Pharmacological Aspects.* Front Pharmacol, 2016. **7**: p. 59.
147. Pathak, A.K., D.C. Jain, and R.P. Sharma, *Chemistry and biological activities of the genera Tinospora.* Int J Pharmacognosy, 1995. **33**(4): p. 277-87.
148. GBIF, *GBIF Backbone Taxonomy*. 2016, GBIF.org: Denmark.
149. Noor, H., et al., *The hypoglycaemic and insulinotropic activity of Tinospora crispa: studies with human and rat islets and HIT-T15 B cells.* Diabetologia, 1989. **32**(6): p. 354-9.
150. Kongsaktrakoon, B., et al., *The antipyretic effect of Tinospora crispa Mier ex. Hock F. & Thoms.* J Pharm Sci, 1994. **21**: p. 1-6.
151. Roosita, K., et al., *Medicinal plants used by the villagers of a Sundanese community in West Java, Indonesia.* J Ethnopharmacol, 2008. **115**(1): p. 72-81.
152. Pachaly, P., A.Z. Adnan, and G. Will, *NMR-Assignments of N-Acylaporphine Alkaloids from Tinospora crispa.* Planta Med, 1992. **58**(2): p. 184-7.
153. Choudhary, M.I., et al., *Alkaloidal constituents of Tinospora crispa.* Nat Prod Commun, 2010. **5**(11): p. 1747-50.
154. Bahari, H., *Anti-atherogenic and antioxidant activities of patawali (Tinospora crispa) on rabbits fed with high cholesterol diet*. 2007, Universiti Putra Malaysia: Malaysia.
155. Aminul, H., I. Ashraful, and S. Mohammad, *Antimicrobial, cytotoxicity and antioxidant activity of Tinospoa crispa.* jpbms, 2011. **13**(12).
156. Ibrahim, M.J., et al., *Anti-proliverative and antioxidant effects of Tinospora crispa (Batawali).* Biomedical Research, 2010. **22**(1).
157. Zulkhairi, A., Jr., et al., *Biological Properties of Tinospora crispa (Akar Patawali) and Its Antiproliferative Activities on Selected Human Cancer Cell Lines.* Malays J Nutr, 2008. **14**(2): p. 173-87.
158. Abood, W.N., et al., *Immunomodulatory effect of an isolated fraction from Tinospora crispa on intracellular expression of INF-gamma, IL-6 and IL-8.* BMC Complement Altern Med, 2014. **14**: p. 205.
159. Najib Nik, A., et al., *Antimalarial activity of extracts of Malaysian medicinal plants.* J Ethnopharmacol, 1999. **64**(3): p. 249-54.
160. Niljan, J., et al., *Antimalarial activity of stem extract of Tinospora crispa against Plasmodium berghei Infection in mice.* J Health Res, 2014. **28**(3).
161. System, U.N.P.G., *GRIN taxonomy: Arcangelisia flava (L.) Merr.* 2017: US.
162. Mandia, E.H., et al., *Arcangelisia flava (L.) Merr*, in *Plant Resources of South-East Asia: Medicinal and poisonous plants*, L.S. de Padua, N. Bunyapraphatsara, and R.H.M.J. Lemmens, Editors. 1999, Backhuys Publisher: Leiden. p. 129-32.
163. Subeki, et al., *Antibabesial activity of protoberberine alkaloids and 20-hydroxyecdysone from Arcangelisia flava against Babesia gibsoni in culture.* J Vet Med Sci, 2005. **67**(2): p. 223-7.
164. Larisu, M.A., *Kajian ilmiah air rebusan batang katola (Arcangelisia falva L. Merr) obat tradisional diare berdarah masyarakat kabupaten Muna Sulawesi Tenggara*. 2011, Universitas Gadjah Mada: Yogyakarta.
165. Keawpradub, N., S. Dej-adisai, and S. Yuenyongsawad, *Antioxidant and cytotoxic activities of Thai medicinal plants named Khaminkhruea: Arcangelisia flava, Coscinium blumeanum and Fibraurea tinctoria.* J Sci Technol, 2005. **27**(2): p. 13.
166. Nguyen-Pouplin, J., et al., *Antimalarial and cytotoxic activities of ethnopharmacologically selected medicinal plants from South Vietnam.* J Ethnopharmacol, 2007. **109**(3): p. 417-427.
167. Sriwilaijareon, N., et al., *Stage specificity of Plasmodium falciparum telomerase and its inhibition by berberine.* Parasitol Int, 2002. **51**(1): p. 99-103.
168. Afrianti, U.R., *Kajian Etnobotani dan Aspek Konservasi Sengkubak [Pycnarrhena cauliflora (Miers.) Diels.] di Kabupaten Sintang Kalimantan Barat*, in *Sekolah Pascasarjana IPB*. 2007, Institut Pertanian Bogor: Bogor.

169. Siwon, J., et al., *Alkaloids from Pycnarrhena longifolia.* Phytochemistry, 1981. **20**(2): p. 3.
170. Masriani, et al., *Pycnarrhena cauliflora ethanolic extract induces apoptosis and cell cycle arrest in hela human cervical cancer cells.* International Journal of Research in Pharmaceutical and Biomedical Sciences, 2013. **4**(4): p. 9.
171. Masriani., E. Eny, and I.K. Adnyana, *Aktivitas antioksidan ekstrak etanol daun sengkubak (Pycnarrhena cauliflora (Miers.) Diels) asal Kalimantan Barat.* 2011.
172. Fricker, S.P., *Metal based drugs: from serendipity to design.* Dalton Trans, 2007(43): p. 4903-17.
173. Bruijnincx, P.C. and P.J. Sadler, *New trends for metal complexes with anticancer activity.* Curr Opin Chem Biol, 2008. **12**(2): p. 197-206.
174. Hambley, T.W., *Developing new metal-based therapeutics: challenges and opportunities.* Dalton Trans, 2007(43): p. 4929-37.
175. Thompson, K.H. and C. Orvig, *Metal complexes in medicinal chemistry: new vistas and challenges in drug design.* Dalton Trans, 2006(6): p. 761-4.
176. Dyson, P.J. and G. Sava, *Metal-based antitumour drugs in the post genomic era.* Dalton Trans, 2006(16): p. 1929-33.
177. Sanchez-Delgado, R.A. and A. Anzellotti, *Metal complexes as chemotherapeutic agents against tropical diseases: trypanosomiasis, malaria and leishmaniasis.* Mini Rev Med Chem, 2004. **4**(1): p. 23-30.
178. Navarro, M., *Gold complexes as potential anti-parasitic agents.* Coordination Chemistry Reviews, 2009. **253**: p. 1619-1626.
179. Navarro, M., H. Perez, and R.A. Sanchez-Delgado, *Toward a novel metal-based chemotherapy against tropical diseases. 3. Synthesis and antimalarial activity in vitro and in vivo of the new gold-chloroquine complex [Au(PPh3)(CQ)]PF6.* J Med Chem, 1997. **40**(12): p. 1937-9.
180. Navarro, M., et al., *The mechanism of antimalarial action of [Au(CQ)(PPh(3))]PF(6): structural effects and increased drug lipophilicity enhance heme aggregation inhibition at lipid/water interfaces.* J Inorg Biochem, 2011. **105**(2): p. 276-82.
181. Lin, J.C., et al., *Coinage metal-N-heterocyclic carbene complexes.* Chem Rev, 2009. **109**(8): p. 3561-98.
182. Teyssot, M.L., et al., *Metal-NHC complexes: a survey of anti-cancer properties.* Dalton Trans, 2009(35): p. 6894-902.
183. Barnard, P.J. and S.J. Berners-Price, *Targeting the mitochondrial cell death pathway with gold compound.* Coord Chem Rev, 2007. **251**: p. 1889-1902.
184. Ray, S., et al., *Anticancer and antimicrobial metallopharmaceutical agents based on palladium, gold, and silver N-heterocyclic carbene complexes.* J Am Chem Soc, 2007. **129**(48): p. 15042-53.
185. Hickey, J.L., et al., *Mitochondria-targeted chemotherapeutics: the rational design of gold(I) N-heterocyclic carbene complexes that are selectively toxic to cancer cells and target protein selenols in preference to thiols.* J Am Chem Soc, 2008. **130**(38): p. 12570-1.
186. Rubbiani, R., et al., *Benzimidazol-2-ylidene gold(I) complexes are thioredoxin reductase inhibitors with multiple antitumor properties.* J Med Chem, 2010. **53**(24): p. 8608-18.
187. Weaver, J., et al., *Cytotoxicity of gold(I) N-heterocyclic carbene complexes assessed by using human tumor cell lines.* Chemistry, 2011. **17**(24): p. 6620-4.
188. Krishnamurthy, D., et al., *Gold(I)-mediated inhibition of protein tyrosine phosphatases: a detailed in vitro and cellular study.* J Med Chem, 2008. **51**(15): p. 4790-5.
189. Özdemir, I., et al., *Synthetic and antimicrobial studies on new gold(I) complexes of imidazolidin-2-ylidenes.* Appl Organomet Chem, 2004. **18**(7): p. 318-22.
190. Hemmert, C., et al., *Synthesis, structures, and antimalarial activities of some silver(I), gold(I) and gold(III) complexes involving N-heterocyclic carbene ligands.* Eur J Med Chem, 2013. **60**: p. 64-75.

191. Micale, N., et al., *Selected gold compounds cause pronounced inhibition of Falcipain 2 and effectively block P. falciparum growth in vitro.* J Inorg Biochem, 2011. **105**(12): p. 1576-9.
192. Trager, W. and J.B. Jensen, *Human malaria parasites in continuous culture.* Science, 1976. **193**(4254): p. 673-5.
193. Benoit-Vical, F., et al., *Trioxaquines are new antimalarial agents active on all erythrocytic forms, including gametocytes.* Antimicrob Agents Chemother, 2007. **51**(4): p. 1463-72.
194. Desjardins, R.E., et al., *Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique.* Antimicrob. Agents Chemother., 1979. **16**: p. 710-718.
195. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
196. Precigout, E., et al., *Analysis of immune responses of different hosts to Babesia divergens isolates from different geographic areas and capacity of culture-derived exoantigens to induce efficient cross-protection.* Infect Immun, 1991. **59**(8): p. 2799-805.
197. Witkowski, B., et al. *Ring-stage Survival Assays (RSA) to evaluate the in-vitro and ex-vivo susceptibility of Plasmodium falciparum to artemisinins*. 2013. **1**.
198. Witkowski, B., et al., *Novel phenotypic assays for the detection of artemisinin-resistant Plasmodium falciparum malaria in Cambodia: in-vitro and ex-vivo drug-response studies.* Lancet Infect Dis, 2013.
199. Angerhofer, C.K., et al., *Antiplasmodial and cytotoxic activity of natural bisbenzylisoquinoline alkaloids.* J Nat Prod, 1999. **62**(1): p. 59-66.
200. Lin, L.Z., et al., *Cytotoxicc and antimalarial bisbenzylisoquinoline alkaloids from Cyclea barbata.* Journal of natural products, 1993. **56-1**: p. 22-29.
201. Fang, D.C. and M.X. Jiang, *Studies on tetrandrine calcium antagonistic action. Chin. Med. J*, 1986. **99**: p. 638-644.
202. Marshall, S.J., et al., *In vitro antiplasmodial, antiamoebic, and cytotoxic activities of a series of bisbenzylisoquinoline alkaloids.* Antimicrob. Agents Chemother., 1994. **38-1**: p. 96-103.
203. Marthianti, A., *The Effects in the Treatment using Tinospora crispa's Stem Extract Compare to Chloroquine on the Amounts of Erythrocyte in the Swiss Mice which was infected with Plasmodium berghei*, in *Faculty of medicine*. 2006, Diponegoro University: Indonesia.
204. Boselli, L., et al., *Synthesis, structures, and selective toxicity to cancer cells of gold(I) complexes involving N-heterocyclic carbene ligands.* Eur J Med Chem, 2014. **85**: p. 87-94.
205. Boselli, L., et al., *Synthesis, structures, and biological study of heterobimetallic Au(I)-Ru(II) complexes involving N-heterocyclic carbene-based multidentate ligands.* Organomettalics, 2015. **34**(6): p. 1046-55.
206. Paloque, L., et al., *Synthesis, characterization, and antileishmanial activities of gold(I) complexes involving quinoline functionalized N-heterocyclic carbenes.* Eur J Med Chem, 2015. **94**: p. 22-9.
207. Amaratunga, C., et al., *Artemisinin resistance in Plasmodium falciparum.* Lancet Infect Dis, 2014. **14**(6): p. 449-50.
208. Nationonline.org, *Political Map of the Republic of Indonesia*. 2016, Nations Online Project.
209. DoH, *Profil Kesehatan Provinsi Nusa Tenggara Timur tahun 2015*. 2016, Department of Health of East Nusa Tenggara Province: Nusa Tanggara Timur.
210. WHO *WHO's emergency response to artemisinin resistance*. 2016. **5**.
211. White, J., 3rd, et al., *In vitro adaptation of Plasmodium falciparum reveal variations in cultivability.* Malar J, 2016. **15**: p. 33.

# ANNEXES

**Annex 1. Plant determination certificate: *T. diversifolia***

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

# CERTIFICATE

Number: 007-02/L-PB/UII/2013

Customer name : Arba Pramundita Ramadani

Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Phone : +62-81804209884

Date of determination : August 1st 2013

The undersigned explained that the plant determination was performed using aerial part of *kembang bulan* in the Laboratory of Pharmaceutical Biology.

The result was :

***Thithonia diversifolia***

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory

LABORATORIUM BIOLOGI FARMASI

**Hady Anshory T, M.Sc.,Apt**

**Annex 2. Plant determination certificate: *C. barbata***

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

# CERTIFICATE

Number: 008-03/L.S-PB/UII/2013

Customer name : Arba Pramundita Ramadani

Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Phone : +62-81804209884

Date of determination : August 1st 2013

The undersigned explained that the plant determination was performed using aerial part of *Cincau* in the Laboratory of Pharmaceutical Biology.

The result was :

***Cyclea barbata***

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory

LABORATORIUM BIOLOGI FARMASI

**Hady Anshory T, M.Sc.,Apt**

**Annex 3. Plant determination certificate: *T. crispa***

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

# CERTIFICATE

Number: 009-03/S-PB/UII/2013

Customer name : Arba Pramundita Ramadani

Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Phone : +62-81804209884

Date of determination : August 3rd 2013

The undersigned explained that the plant determination was performed using aerial part of *Brotowali* in the Laboratory of Pharmaceutical Biology.

The result was :

***Tinospora crispa* (L.) Miers**

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory

LABORATORIUM BIOLOGI FARMASI

**Hady Anshory T, M.Sc.,Apt**

**Annex 4. Plant determination certificate: *A. flava***

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

# CERTIFICATE

Number: 010-04/S-PB/UII/2013

Customer name : Arba Pramundita Ramadani

Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Phone : +62-81804209884

Date of determination : August 3rd 2013

The undersigned explained that the plant determination was performed using lignum part of *Kayu kuning* in the Laboratory of Pharmaceutical Biology.

The result was :

***Arcangelisia flava***

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory

**Hady Anshory T, M.Sc.,Apt**

**Annex 5. Plant determination certificate: *P. cauliflora***

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

# CERTIFICATE

Number: 011-01/S.R-PB/UII/2013

Customer name : Arba Pramundita Ramadani

Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Phone : +62-81804209884

Date of determination : August 3rd 2013

The undersigned explained that the plant determination was performed using aerial part of *Sengkubak* in the Laboratory of Pharmaceutical Biology.

The result was :

***Pycnarrhena cauliflora* (Miers.) Diels**

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory

UNIVERSITAS ISLAM INDONESIA LABORATORIUM BIOLOGI FARMASI FAKULTAS

**Hady Anshory T, M.Sc.,Apt**

**Annex 6. Supplementary materials for publication of K13**

www.sciencemag.org/cgi/content/full/science.1260867/DC1

# Supplementary Materials for

## K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates

Judith Straimer, Nina F. Gnädig, Benoit Witkowski, Chanaki Amaratunga, Valentine Duru, Arba P. Ramadani, Mélanie Dacheux, Nimol Khim, Lei Zhang, Stephen Lam, Philip D. Gregory, Fyodor D. Urnov, Odile Mercereau-Puijalon, Françoise Benoit-Vical, Rick M. Fairhurst, Didier Ménard, David A. Fidock*

*Corresponding author. E-mail: df2260@columbia.edu

Published 11 December 2014 on *Science* Express
DOI: 10.1126/science.1260867

**This PDF file includes:**

Materials and Methods
Figs. S1 to S4
Tables S1 to S5
Statistical data
References

## MATERIALS AND METHODS

**Zinc-finger nuclease (ZFN) engineering.** ZFNs directed against *K13* (PlasmoDB ID PF3D7_1343700; also referred to as Kelch13) were designed and assembled using an archive of validated modules (*33*). These were screened for activity using an episomal reporter system, yielding ZFN pairs 18/19 and 18/20 (**Table S1**).

**Plasmid construction.** Left and right ZFNs were first linked with the 2A peptide to ensure equal levels of expression driven by a single promoter (*20*). This ZFN L-2A-ZFN R sequence was cloned into the AvrII and XhoI restriction sites in pDC2 (*34*). This placed the fused sequence under the regulatory control of the 5' *calmodulin* (PF3D7_1434200) promoter and the 3' *hsp86* (PF3D7_0708500) terminator. This plasmid also contained the human *dhfr* selectable marker (**Fig. S1**).

We generated six different donor plasmids to introduce either the wild-type *K13* allele or one of five mutant alleles: M476I (allele 1), C580Y (allele 2), R539T (allele 3), Y493H (allele 4) or I543T (allele 5). In a first step we amplified a 1.5-kb donor sequence of the *K13*-propeller domain using the primer pair p1/p2 (**Table S5**) and cloned this into the pGEM®-T vector (Promega). At the zinc-finger binding site we introduced six silent mutations by site-directed mutagenesis using the primer pair p3/p4. Allele-specific mutations were introduced via mutagenesis with specific primer pairs: p5/p6 for M476I, p7/p8 for C580Y, p9/p10 for R539T, p11/p12 for Y493H and p13/p14 for I543T. *K13* donor sequences were excised from the pGEM-T vector with the restriction enzymes SacII and PstI while the ZFN-containing plasmid was digested with SalI and PstI. The two incompatible sites SacII and SalI from the donor fragment and the vector, respectively, were previously filled in using Klenow DNA polymerase. *K13* templates were ligated into the pDC2-based plasmids described above (with primer pairs 18/19 or 18/20, both showing similar efficiency; **Table S1**) using the compatible PstI site and blunt-end cloning, yielding the final pZFN$^{K13}$-h*dhfr* transfection plasmids.

**Parasite cultures, transfections and DNA analysis.** Asexual blood-stage parasites were propagated in human erythrocytes in RPMI-1640 medium containing 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mM HEPES, 0.225% $NaHCO_3$, 10 mg/L gentamycin and 0.5% (w/v) Albumax II (Invitrogen) for the Dd2, FCB and V1/S reference lines. Cambodian isolates and F32 parasites were further supplemented with 5% human AB$^+$ serum (Interstate Blood Bank). Parasites were maintained at 37°C in an atmosphere

of 5% $O_2$, 5% $CO_2$ and 90% $N_2$, and electroporated with purified circular plasmid DNA as described (*35*). One day after electroporation, parasites were exposed to 2.5 nM or 10 nM WR99210 for 6 days (the latter concentration was reserved for quadruple *dhfr* mutant parasites; **Table S3**) to select for transformed parasites (**Fig. S1A**). Parasites generally became microscopically detectable 15–25 days post-electroporation. *K13* editing was assessed by PCR analysis of bulk cultures, and cultures showing the highest level of editing were selected for 96-well cloning by limiting dilution (**Table S2**). Clones were identified after 17–28 days by staining with 2× SYBR Green I and 1.6 μM MitoTracker Deep Red (Invitrogen) and assaying for growth by flow cytometry on an Accuri C6 cytometer (*36*). To test for *K13* editing, *P. falciparum* trophozoite-infected erythrocytes were harvested and saponin-lysed. Parasite genomic DNA was extracted and purified using DNeasy Blood kits (Qiagen). *K13* sequences (**Fig. 1**) were examined by PCR-amplifying the genomic locus with primers p15/p16 flanking the *K13* plasmid donor sequence. These products were amplified from bulk cultures or parasite clones. Sequencing was performed with the internal p17 primer (**Table S5**). For select clones, expression of the mutant or wild-type *K13* alleles was confirmed by RT-PCR and sequencing.

**Ring-stage survival assays (RSA$_{0\text{-}3h}$).** These assays were carried out as previously described, with minor modifications (*12*). A detailed protocol is available on the WorldWide Antimalarial Resistance Network's website at http://www.wwarn.org/toolkit/procedures/ring-stage-survival-assays-rsa-evaluate-susceptibility-p-falciparum. In summary, 10–15 mL parasite cultures were synchronized 1–2 times using 5% sorbitol (Sigma-Aldrich). Synchronous multinucleated schizonts were incubated in RPMI-1640 containing 15 units/ml sodium heparin for 15 min at 37°C to disrupt agglutinated erythrocytes, concentrated over a gradient of 75% Percoll (Sigma-Aldrich), washed once in RPMI-1640, and incubated for 3 hours with fresh erythrocytes to allow time for merozoite invasion. Cultures were then subjected again to sorbitol treatment to eliminate remaining schizonts. The 0–3 hours post-invasion rings were adjusted to 1% parasitemia and 2% hematocrit in 1 mL volumes (in 48-well plates), and exposed to 700 nM DHA or 0.1% DMSO (solvent control) for 6 hours. Duplicate wells were established for each parasite line ± drug. One-mL cultures were then transferred to 15 mL conical tubes, centrifuged at 800xg for 5 min to pellet the cells, and the supernatants carefully removed. As a washing step to remove drug, 9 mL culture medium were added to each tube and the cells resuspended, centrifuged, and the medium aspirated. Fresh medium lacking drug was then added to cultures, which were returned to standard culture conditions for 66 hours.

Parasite viability was assessed by microscopic examination of Giemsa-stained thin blood smears by counting parasites that developed into second-generation rings or trophozoites with normal morphology. To obtain a homogenous smear for all slides, the cultures were resuspended, transferred into an Eppendorf tube, and briefly centrifuged. 2 μL of the pellet were then used for each smear. Parasitemias were calculated from a total of at least 40,000 erythrocytes per assay. Slides were read from the two duplicate wells per assay by two separate microscopists, each of whom examined at least 10,000 erythrocytes per slide. In instances of >20% discrepancy in parasite counts, slides were further examined by a third microscopist. The mean±SEM numbers of erythrocytes counted per parasite line and per assay were 44,160±970. Our mean±SEM parasitemia for non DHA-treated cultures was 5.1±0.3%. For assays where ≤5 infected erythrocytes were counted from the initial examination of 40,000 erythrocytes (corresponding to a calculated parasitemia of ≤0.0125%), the total number of erythrocytes was expanded to a mean±SEM of 58,360±3,460.

Percent survival was calculated as the parasitemia in the drug-treated sample divided by the parasitemia in the untreated sample ×100. As an example, a 20% $RSA_{0\text{-}3h}$ survival value corresponded to a 1% parasitemia in drug-treated parasites compared to a 5% parasitemia in the untreated control (the mean parasitemia in our untreated controls). Assuming a total cell count of 40,000 erythrocytes, this produced a 95% confidence interval (CI) of 0.9–1.1% parasitemia (corresponding to 360–440 infected erythrocytes for that particular line in one assay). A 2% $RSA_{0\text{-}3h}$ survival value corresponded to a 0.1% parasitemia in the drug-treated sample (95% CI 0.07–0.13%; i.e. 28–52 infected erythrocytes) assuming a count of 40,000 erythrocytes. Given a 0.01% parasitemia (corresponding to a 0.2% $RSA_{0\text{-}3h}$ survival value) and an average count of 58,360 erythrocytes, the 95% CI was 0.002–0.018% (i.e. 1–10 infected erythrocytes). The limit of assay sensitivity was therefore set to 0.2% survival. For each parent and corresponding *K13*-edited clones, individual groups were designated to perform the $RSA_{0\text{-}3h}$ assays, whose results are presented in Fig. 2 and summarized in Table S4.

***In vitro* 50% inhibitory concentration ($IC_{50}$) assays.** *In vitro* $IC_{50}$ values were determined by incubating parasites for 72 hours across a range of concentrations of DHA or artesunate (0.25–24 nM for both). Proliferation was determined by staining parasites with SYBR Green I and MitoTracker Deep Red, and measuring parasitemia by flow cytometry (see above). *In vitro* $IC_{50}$ values were calculated by nonlinear regression analysis, and Student *t* tests were used for statistical analysis.

**Fig. S1**

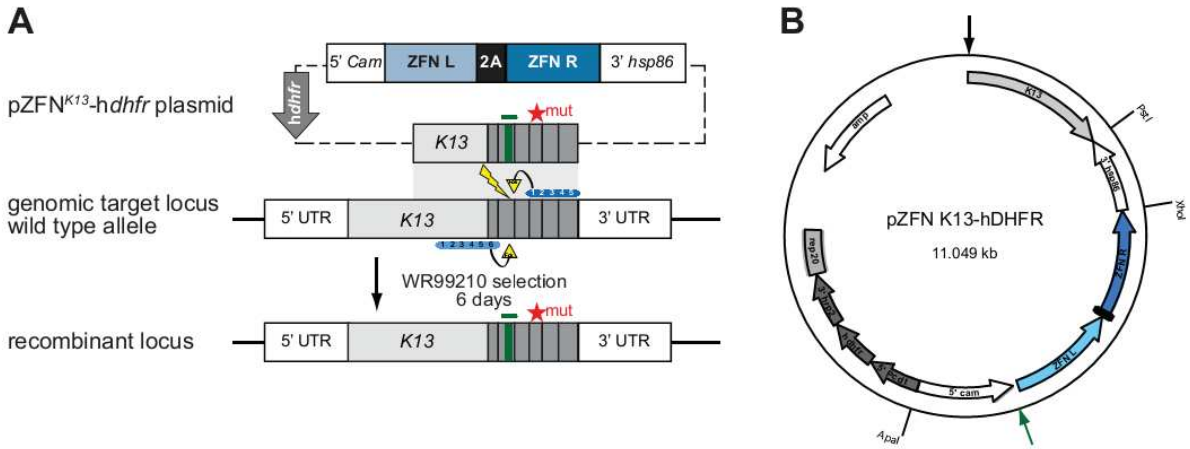



**Fig. S1. *K13*-propeller editing strategy and plasmid map. (A)** SNPs were inserted into wild-type or removed from mutant *K13*-propeller alleles using zinc-finger nucleases (ZFNs; **Table S1**). In this schematic, ZFNs introduce a double-stranded break (thunderbolt) in the 2nd propeller domain of the wild-type genomic target locus, thus triggering DNA resection. The pZFN$^{K13}$-h*dhfr* donor plasmid sequence provides a DNA template for homology-directed repair processes. Green bars indicate the presence of silent binding-site mutations that prevent the plasmid and the edited recombinant locus from being cleaved by ZFNs. Red stars indicate the presence of a non-synonymous propeller mutation in the plasmid and edited recombinant locus. **(B)** pZFN$^{K13}$-h*dhfr* plasmid map. Plasmids contained either the ZFN pairs 18/20 or 19/20 (**Table S1**). Black and green arrows indicate SacII/SalI and AvrII/NheI restriction sites that were ablated during plasmid construction (see supplementary text).

**Fig. S2**

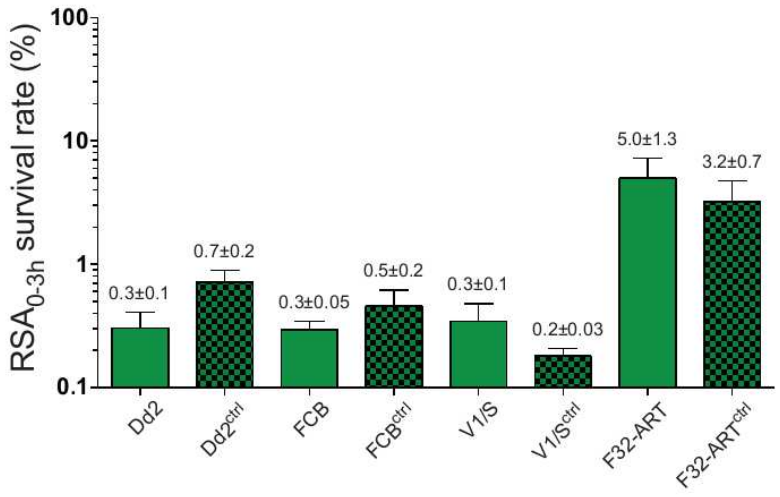



**Fig. S2. Synonymous *K13* binding-site mutations do not significantly alter ring-stage susceptibility to artemisinins.** Parasite lines were transfected with pZFN$^{K13}$-h*dhfr* plasmids harboring only silent binding-site mutations in the *K13* donor template (**Fig. 1**), and edited clones were obtained. $RSA_{0\text{-}3h}$ assays were performed on 3–5 separate occasions in duplicate, and percent survival was calculated as described in Methods. We note that Dd2, FCB and V1/S harbor the wild-type *K13* allele, whereas F32-ART harbors the K13-propeller mutation M476I that was acquired during several years of artemisinin pressure *in vitro*. The F32-ART line and the edited F32-ART$^{ctrl}$ line carrying silent binding-site mutations (in addition to M476I), showed survival rates in the $RSA_{0\text{-}3h}$ that were 2–3 fold lower than those originally reported for F32-ART, suggesting that the resistance phenotype had attenuated over time. Mean±SEM values are represented above each column. Student *t* tests revealed no significant differences between the edited clones harboring only the synonymous binding-site mutations (designated with superscript ctrl) and the isogenic parental lines ($p$>0.05 in all cases).

**Fig. S3**

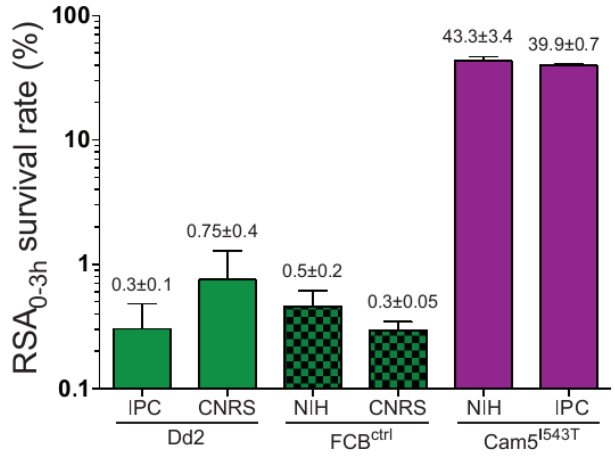



**Fig. S3. Demonstration of consistent $RSA_{0\text{-}3h}$ values between test sites.** To test for consistency between research groups, several parasite lines were shipped to separate laboratories for independent analysis. Results showed equivalent survival rates among the three sites, namely those led by Dr. Didier Ménard at the Institut Pasteur du Cambodge (IPC), Dr. Françoise Benoit-Vical at the Université de Toulouse, Centre National de Recherche Scientifique UPR8241 (CNRS), and Dr. Rick Fairhurst at the National Institutes of Health (NIH). Assays were performed on 2–3 separate occasions in duplicate, and results are shown as mean±SEM above each column. Dd2 and $FCB^{ctrl}$ parasites harbor the wild-type *K13* allele (the latter parasite line also contains synonymous binding-site mutations), whereas Cam5 parasites harbor the K13 I543T mutation that confers elevated survival rates in the $RSA_{0\text{-}3h}$, indicative of high-level *in vitro* resistance. *t* tests showed no significant difference between $RSA_{0\text{-}3h}$ results of different laboratories when testing the same parasite lines ($p>0.05$ in all cases).

Fig. S4

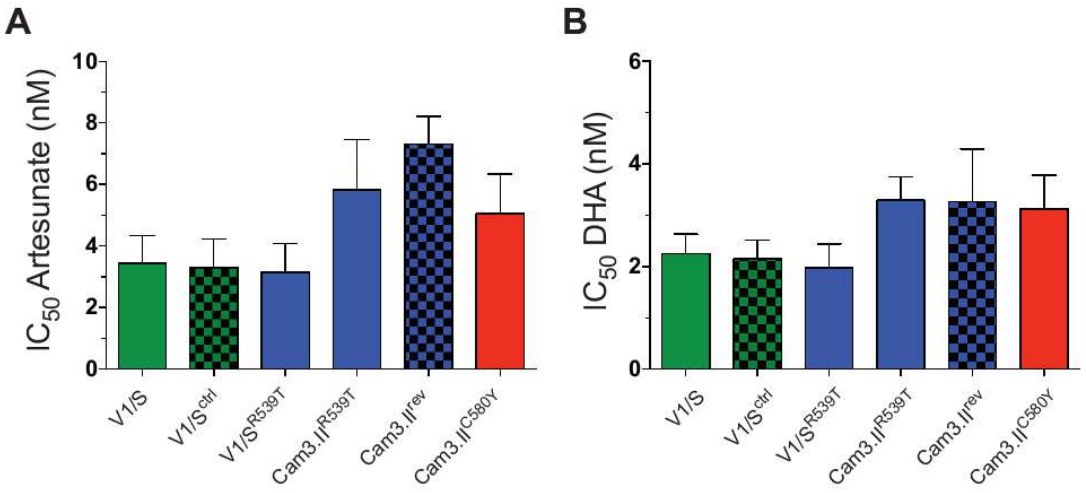



**Fig. S4. *In vitro* proliferation assays show no significant differences in $IC_{50}$ values between K13 wild-type and mutant parasites.** $IC_{50}$ values (mean±SEM) were measured in 72-h proliferation assays with final parasitemias determined using flow cytometry with SYBR Green I and MitroTracker Deep Red-stained parasites. Assays were performed on three separate occasions in duplicate. DHA, dihydroartemisinin.

**Table S1. Nucleotide sequences of *K13*-specific ZFN binding sites and amino acid sequences of zinc-finger proteins.**

| *K13* ZFN Binding Sequence (underlined) | ZFN | Finger 1 | Finger 2 | Finger 3 | Finger 4 | Finger 5 | Finger 6 |
|---|---|---|---|---|---|---|---|
| CTTTGACTCCACATACTAGCAAATTCTCTACATACCATACAAAGT | 43418-ELD[a] | RSDNLSV | IRSTLRD | RSADLTR | TNQNRIT | TSGNLTR | DSSNLAT |
| GAAACTGAGGTGTATGATCGTTTAAGAGATGTATGGTATGTTTCA | 43419-KKR | TSGSLTR | TSSNRKT | RPTRMQ | QSGSLTR | TSANLSR | - |
| GAAACTGAGGTGTATGATCGTTTAAGAGATGTATGGTATGTTTCA | 43420-KKR | TSGSLSR | TSSNRRH | RSAHLTT | QSGSLTR | TSANLSR | - |

Amino acid sequences of ZFNs used in this study (individual fingers are underlined).

43418-ELD (414 amino acids)
MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAAMAERPFQCRICMRNFSRSDNLSVHIRTHTGEKPFACDICGRKFAIRSTLRDHTKIHTGSQKPFQCRICMRNFSRSADL
TRHIRTHTGEKPFACDICGRKFATNQNRITHTKIHTGSQKPFQCRICMRNFSTSGNLTRHIRTHTGEKPFACDICGRKFADSSNLATHTKIHLRGSQLVKSELEEKKSELRHKLKYVP
HEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMERYVEENQTRDKHLNPNEWWKVYPSSVTEFKFLFVSGH
FKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINFRSXX

43419-KKR (382 amino acids)
MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAAMAERPFQCRICMRKFATSGSLTRHTKIHTGEKPFQCRICMRNFSTSSNRKTHIRTHTGEKPFACDICGRKFARPTRMQ
HTKIHTGSQKPFQCRICMRNFSQSGSLTRHIRTHTGEKPFACDICGRKFATSANLSRHTKIHLRGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKV
YGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVKENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNRKTNCNGAVLSVEELLI
GGEMIKAGTLTLEEVRRKFNNGEINFXX

43420-KKR (383 amino acids)
MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAAMAERPFQCRICMRNFSTSGSLSRHIRTHTGEKPFACDICGRKFATSSNRRHHTKIHTGSQKPFQCRICMRKFARSAHL
TTHTKIHTGEKPFQCRICMRNFSQSGSLTRHIRTHTGEKPFACDICGRKFATSANLSRHTKIHLRGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMK
VYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVKENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNRKTNCNGAVLSVEELL

[a] The left ZFN 43418-ELD contains six zinc fingers, whereas the two right ZFNs 43419-KKR and 43420-KKR each contain five. Each finger recognizes a 3-nucleotide *K13* sequence. 43418-ELD binds to the *K13* non-coding (-) strand, whereas 43419-KKR and 43420-KKR bind to the same sequence on the coding (+) strand. 43418-ELD / 43419-KKR, and 43418-ELD / 43420-KKR constitute the ZFN pairs 18/19 and 18/20 respectively (Table S3).

**Table S2. Geographic origin, native *K13* allele and drug-resistance genotypes of *Plasmodium falciparum* clinical isolates and reference lines.**

| Parasite | Original ID | Sanger ID | Provider | Geographic origin (year) | $PCt_{1/2}$ (h) | KH group | K13-propeller | PMID # | *pfcrt* | *pfmdr1* | *pfmdr1* CN | *dhfr* | *dhps* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cam3.I | IPC 5202 | N/A | D. Ménard | Battambang, W. Cambodia (2011) | ND | ND | R539T (allele 3) | 24352242 | Dd2[a] | 184F | 4 | Triple[b] | 436A/540E |
| Cam3.II | RF 967 | PH0306-C | R. Fairhurst | Pursat, W. Cambodia (2010) | 6.0 | 3 | R539T (allele 3) | 22940027 | Dd2 | 184F | 1 | Triple | 436A/540E |
| Cam5 | IPC 4912 | N/A | D. Ménard | Mondulkiri, E. Cambodia (2011) | ND | ND | I543T (allele 5) | 24352242 | Dd2 | WT | 1 | Quadruple | 436F/540E/613S |
| Cam2 | IPC 3445 | N/A | D. Ménard | Pailin, W. Cambodia (2010) | ND | ND | C580Y (allele 2) | 24352242 | Dd2 | WT | 2 | Triple | 436A/540E |
| CamWT | RF 915 | PH0164-C | R. Fairhurst | Pursat, W. Cambodia (2010) | 3.7 | 1 | Wild-type | 22940027 | Dd2 | WT | 1 | Triple | 436A/540E |
| F32-TEM | F32 | N/A | F. Benoit-Vical | Tanzania (1982) | N/A | N/A | Wild-type | 20160056 | WT | WT | 1 | WT | 436A |
| V1/S | V1/S | N/A | MR4 | Vietnam (1976) | N/A | N/A | Wild-type | 12124623 | Dd2 | 86Y | 1 | Quadruple | 613T |
| FCB | FCB | N/A | MR4 | Asia (1980) | N/A | N/A | Wild-type | 12124623 | FCB | 86Y | 2 | 16V/108T | 437A |
| Dd2 | Dd2 | N/A | MR4 | Indochina (1980) | N/A | N/A | Wild-type | 12124623 | Dd2 | 86Y | 3 | Triple | 436F/613S |

[a] Dd2: 74I/75D/76T/220S/271E/326S/356T/371I; FCB: 74I/75E/76T/220S/271E/326S/371I.

[b] Triple, 51I/59R/108N; Quadruple, 51I/59R/108N/164L.

$PCt_{1/2}$, parasite clearance half-life; PMID #, PubMed identification number; *pfmdr1* CN, *pfmdr1* copy number; MR4, Malaria Research and Reference Reagent Resource, Manassas, VA; N/A, not available; ND, not determined.

**Table S3. *K13* transfection outcomes with *Plasmodium falciparum* clinical isolates and reference lines.**

| | | Binding site mutations only | | M476I (allele 1) | | C580Y (allele 2) | | R539T (allele 3) | | Y493H (allele 4) | | I543T (allele 5) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Parasite** | **ZFN pair** | Edited cultures (%) | Edited clones (%) | Edited cultures (%) | Edited clones (%) | Edited cultures (%) | Edited clones (%) | Edited cultures (%) | Edited clones (%) | Edited cultures (%) | Edited clones (%) | Edited cultures (%) | Edited clones (%) |
| Cam3.I | 18/19 | 1 of 1 (100%) | ND | ND | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| Cam3.I | 18/20 | 1 of 1 (100%) | 2 of 7 (29%) a | ND | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| Cam3.II | 18/19 | 1 of 2 (50%) | 4 of 16 (25%) | ND | N/A | 0 of 1 (0%) | ND | ND | N/A | ND | N/A | ND | N/A |
| Cam3.II | 18/20 | 1 of 2 (50%) | ND | ND | N/A | 1 of 1 (100%) | 1 of 4 (25%) | ND | N/A | ND | N/A | ND | N/A |
| Cam5 | 18/19 | 1 of 4 (25%) | ND | ND | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| Cam5 | 18/20 | 1 of 4 (25%) | 6 of 11 (55%) | ND | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| Cam2 | 18/19 | 1 of 2 (50%) | 1 of 6 (17%) | ND | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| Cam2 | 18/20 | 1 of 2 (50%) | ND | ND | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| CamWT | 18/19 | 0 of 1 (0%) | ND | ND | N/A | 1 of 2 (50%) | 1 of 2 (50%) | 0 of 1 (0%) | ND | 0 of 2 (0%) | ND | 1 of 2 (50%) | 0 of 3 (0%) |
| CamWT | 18/20 | ND | N/A | ND | N/A | 0 of 2 (0%) | ND | 0 of 1 (0%) | ND | 1 of 2 (50%) | ND | ND | N/A |
| F32-TEM | 18/19 | ND | N/A | 1 of 2 (50%) | ND | ND | N/A | ND | N/A | ND | ND | ND | N/A |
| F32-TEM | 18/20 | ND | N/A | 1 of 2 (50%) | 2 of 19 (11%) | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| F32-ART | 18/19 | 1 of 2 (50%) | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| F32-ART | 18/20 | 1 of 2 (50%) | 0 of 3 (0%) b | ND | N/A | ND | N/A | ND | N/A | ND | N/A | ND | N/A |
| V1/S | 18/19 | 0 of 1 (0%) | N/A | 0 of 2 (0%) | ND | 1 of 1 (100%) | 0 of 18 (0%) | 0 of 1 (0%) | ND | 1 of 2 (50%) | ND | 0 of 1 (0%) | ND |
| V1/S | 18/20 | 1 of 1 (100%) | ND c | 0 of 2 (0%) | ND | 0 of 1 (0%) | ND | 1 of 1 (100%) | 1 of 8 (13%) | 0 of 1 (0%) | ND | 0 of 1 (0%) | ND |
| FCB | 18/19 | 0 of 1 (0%) | N/A | 0 of 1 (0%) | ND | 1 of 2 (50%) | 2 of 25 (8%) | 0 of 3 (0%) | ND | 0 of 2 (0%) | ND | 0 of 2 (0%) | ND |
| FCB | 18/20 | 0 of 1 (0%) | N/A | 0 of 1 (0%) | ND | 0 of 2 (0%) | ND | 0 of 3 (0%) | ND | 0 of 3 (0%) | ND | 0 of 2 (0%) | ND |
| Dd2 | 18/19 | 0 of 1 (0%) | N/A | 0 of 3 (0%) | ND | 1 of 1 (100%) | 1 of 10 (10%) | 0 of 6 (0%) | ND | 1 of 1 (100%) | 1 of 17 (6%) | 0 of 3 (0%) | ND |
| Dd2 | 18/20 | 1 of 3 (33%) | ND | 1 of 3 (33%) | 1 of 8 (13%) | 0 of 1 (0%) | ND | 1 of 6 (0%) | 1 of 8 (13%) | 0 of 1 (0%) | ND | 1 of 3 (33%) | 3 of 21 (14%) |
| Positive events | | 12 of 30 (40%) d | 13 of 43 (30%) | 3 of 16 (19%) | 3 or 27 (11%) | 5 of 14 (36%) e | 5 of 59 (9%) | 2 of 22 (9%) | 2 of 16 (13%) | 3 of 14 (21%) | 1 of 17 (6%) | 2 of 14 (14%) | 3 of 24 (13%) |

[a] Clones were generated from individual bulk cultures with the highest percentage of editing events for a given mutation and parasite.The percentages of edited clones are listed only for those that captured the desired nucleotide changes (either insertion or removal of a point mutation of interest). Clones that captured binding site mutations but not *K13* mutations were not included in this analysis; therefore, the percentages listed underestimate the percentage of editing events.

[b] One clone (F32-ART$^{ctrl}$ in Fig. S3) captured the binding site mutations, but did not revert M476I to wild-type. This clone was therefore not listed as being edited at the mutation of interest (see definition in footnote a).

[c] This culture was not cloned because the desired binding site control line (V1/S$^{ctrl}$) was obtained from the transfection with the R539T plasmid, resulting in incorporation of the binding site mutations but not R539T. This was one of several instances where a binding site mutant control was obtained from transfections with mutant donor sequences that captured only the binding site mutations, but not the *K13* mutations more distant from the ZFN cleavage site. Binding site control clones are shown in Fig. S3.

[d] These data suggest that removing K13 mutations (achieved using the plasmid containing the binding site mutations only) was generally more efficient than introducing them, possibly due to a fitness cost associated with their presence.

[e] These data suggest that C580Y editing into K13 wild-type parasites was more efficient than editing of other mutations, possibly because the C580Y mutation might have a lesser fitness cost. These results are consistent with C580Y being highly prevalent in western Cambodia, despite our evidence that C580Y mediates a lower level of *in vitro* resistance than R539T or I543T (see Fig. 2).
N/A, not applicable; ND, not done.

**Table S4. Data from ring-stage survival assays (RSAs).**

| Parasite | Original isolate/line | K13 sequence | Binding-site mutations | RSA values mean ± SEM | Assays | Primary site of RSA data |
|---|---|---|---|---|---|---|
| Cam3.I$^{R539T}$ | IPC 5202 | R539T (parent allele 3) | No | 40.2 ± 3.0[a,b] | 3[c] | IPC, Cambodia[d] |
| Cam3.I$^{rev}$ | IPC 5202 | wild-type (revertant) | Yes | 0.3 ± 0.1 | 3 | IPC, Cambodia |
| Cam3.II$^{R539T}$ | RF 967 | R539T (parent allele 3) | No | 48.9 ± 0.8 | 3 | NIH, USA |
| Cam3.II$^{rev}$ | RF 967 | wild-type (revertant) | Yes | 0.7 ± 0.2 | 3 | NIH, USA |
| Cam3.II$^{C580Y}$ | RF 967 | C580Y (allele 2) | Yes | 24.1 ± 2.9 | 3 | NIH, USA |
| Cam5$^{I543T}$ | IPC 4912 | I543T (parent allele 5) | No | 43.4 ± 3.4 | 3 | NIH, USA |
| Cam5$^{rev}$ | IPC 4912 | wild-type (revertant) | Yes | 0.3 ± 0.02 | 3 | NIH, USA |
| Cam2$^{C580Y}$ | IPC 3445 | C580Y (parent allele 2) | No | 13.0 ± 2.6 | 3 | IPC, Cambodia |
| Cam2$^{rev}$ | IPC 3445 | wild-type (revertant) | Yes | 2.4 ± 0.1 | 3 | IPC, Cambodia |
| CamWT | RF 915 | wild-type (parent) | No | 0.6 ± 0.1 | 3 | NIH, USA |
| CamWT$^{C580Y}$ | RF 915 | C580Y (allele 2) | Yes | 8.9 ± 0.5 | 3 | NIH, USA |
| F32-TEM | F32-TEM | wild-type (parent) | No | <0.2[e] | 4 | CNRS, France |
| F32-TEM$^{M476I}$ | F32-TEM | M476I (allele 1) | Yes | 1.7 ± 0.4 | 4 | CNRS, France |
| V1/S | V1/S | wild-type (parent) | No | 0.3 ± 0.1 | 3 | NIH, USA |
| V1/S$^{R539T}$ | V1/S | R539T (allele 3) | Yes | 20.7 ± 4.0 | 4 | NIH, USA |
| FCB | FCB | wild-type (parent) | No | 0.3 ± 0.1 | 3 | NIH, USA |
| FCB$^{C580Y}$ | FCB | C580Y (allele 2) | Yes | 1.9 ± 0.3 | 4 | NIH, USA |
| Dd2 | Dd2 | wild-type (parent) | No | 0.3 ± 0.1 | 3 | IPC, Cambodia |
| Dd2$^{Y493H}$ | Dd2 | Y493H (allele 4) | Yes | 1.7 ± 0.4 | 3 | IPC, Cambodia |
| Dd2$^{C580Y}$ | Dd2 | C580Y (allele 2) | Yes | 4.1 ± 0.4 | 3 | IPC, Cambodia |
| Dd2$^{M476I}$ | Dd2 | M476I (allele 1) | Yes | 9.8 ± 2.6 | 4 | CNRS, France |
| Dd2$^{R539T}$ | Dd2 | R539T (allele 3) | Yes | 19.4 ± 0.8 | 3 | NIH, USA |
| Dd2$^{I543T}$ | Dd2 | I543T (allele 5) | Yes | 29.1 ± 3.9 | 3 | IPC, Cambodia |

[a] RSA values are the percentage of parasites that survived a 6-h pulse of 700 nM dihydroartemisinin, compared to DMSO-treated parasites. Parasitemias were determined 66 h after ending the drug pulse.

[b] For each RSA value, the relative standard error (RSE) was calculated as the SEM/mean x100. The mean RSE for all assays was 17.0%, indicating an average SEM of 17.0% of the mean.

[c] Assays were performed on separate occasions as enumerated, with each assay performed in duplicate.

[d] IPC, Institut Pasteur du Cambodge (Ménard lab); CNRS, Centre National de Recherche Scientifique UPR8241 (Benoit-Vical lab); NIH, National Institutes of Health (Fairhurst lab).

[e] RSA values for F32-TEM were calculated to be 0.02±0.02. Based on our limit of sensitivity (as discussed in Supplementary Materials) we have indicated this survival value to be <0.2%.

**Table S5. Oligonucleotides used in this study.**

| Name | Nucleotide sequence (5'-3')[a] | Description | Lab name |
|---|---|---|---|
| p1 | GTGACGTCGATTGATATTAATGTTGGTGGAGC | *K13* donor fwd | p3984 |
| p2 | CCGCATATGGTGCAAACGGAGTGACCAAATCTGGG | *K13* donor rev | p3986 |
| p3 | GGTGTATGATaGaTTAAGAGAcGTcTGGTATGTaTCAAGTAATTTAAATATACC | SDM ZFN binding site mutation fwd | p4169 |
| p4 | CCAgACgTCTCTTAAtCtATCATACACCTCtGTTTCAAATAAAGCC | SDM ZFN binding site mutation rev | p4170 |
| p5 | GCTGGCGTATGTGTACACCTATaTCTACCAAAAAAGCTTATTTTGGAAGTGC | SDM allele 1 M476I fwd | p4173 |
| p6 | GGTAGAtATAGGTGTACACATACGCCAGCATTGTTGACTAATATCTAATAATTCC | SDM allele 1 M476I rev | p4174 |
| p7 | CATCAGCTATGTaTGTTGCTTTTGATAATAAAATTTATGTCATTGG | SDM allele 2 C580Y fwd | p4003 |
| p8 | ATTATCAAAAGCAACAtACATAGCTGATGATCTAGGGGTATTCAAAGG | SDM allele 2 C580Y rev | p4004 |
| p9 | CGTCAAATGGTAcAATTTATTGTATTGGGGGATATGATGGCTCTTC | SDM allele 3 R539T fwd | p4005 |
| p10 | CCAATACAATAAATTgTACCATTTGACGTAACACCACAATTATTTC | SDM allele 3 R539T rev | p4006 |
| p11 | GAATAATTTCTTAcACGTTTTTGGTGGTAATAACTATGATTATAAGGC | SDM allele 4 Y493H fwd | p4188 |
| p12 | CCAAAAACGTgTAAGAAATTATTCAATACAGCACTTCCAAAATAAGC | SDM allele 4 Y493H rev | p4189 |
| p13 | GGTAGAATTTATTGTAcTGGGGGATATGATGGCTCTTCTATTATACC | SDM allele 5 I543T fwd | p4007 |
| p14 | CATATCCCCCAgTACAATAAATTCTACCATTTGACGTAACACCAC | SDM allele 5 I543T rev | p4008 |
| p15 | GCAAATCTTATAAATGATGATTCTGG | *K13* 5' for screening fwd | p4433 |
| p16 | GCTAATAAGTAATATCAATATAAGGG | *K13* 3' for screening rev | p4434 |
| p17 | GGTATTAAATTTTTACCATTCCCATTAGTATTTTGTATAGG | Sequencing primer | p4186 |

[a] Lower-case nucleotides show the position that was subjected to mutagenesis.

fwd, forward; rev, reverse; SDM, site directed mutagenesis.

## Statistical data: Results of two-sample t-tests with unequal variances

### Cam3.I$^{R539T}$ vs. Cam3.I$^{rev}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2A)

```
. ttest logCam3I, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | 3.688271 | .0768044 | .133029 | 3.357809 | 4.018734 |
| 2 | 3 | -1.546587 | .4405906 | .7631253 | -3.442296 | .3491212 |
| combined | 6 | 1.070842 | 1.187515 | 2.908805 | -1.981761 | 4.123445 |
| diff | | 5.234858 | .4472348 | | 3.412362 | 7.057355 |

```
    diff = mean(1) - mean(2)                                      t =  11.7049
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.12144

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9971         Pr(|T| > |t|) = 0.0058          Pr(T > t) = 0.0029
```

### Cam3.II$^{R539T}$ vs. Cam3.II$^{rev}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2B)

```
. ttest logCam3II, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | 3.89019 | .016374 | .0283606 | 3.819738 | 3.960642 |
| 2 | 3 | -.5074539 | .3730999 | .646228 | -2.112773 | 1.097866 |
| combined | 6 | 1.691368 | .9974256 | 2.443184 | -.8725962 | 4.255332 |
| diff | | 4.397644 | .3734591 | | 2.796674 | 5.998614 |

```
    diff = mean(1) - mean(2)                                      t =  11.7754
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.0077

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9965         Pr(|T| > |t|) = 0.0070          Pr(T > t) = 0.0035
```

## $Cam5^{I543T}$ vs. $Cam5^{rev}$ (groups 1 vs. 2 respectively; $p$=<0.0001; Fig. 2C)

```
. ttest logCam5, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | 3.763479 | .0753753 | .1305539 | 3.439165 | 4.087793 |
| 2 | 3 | -1.215887 | .0725464 | .125654 | -1.528029 | -.9037451 |
| combined | 6 | 1.273796 | 1.114403 | 2.729718 | -1.590867 | 4.138459 |
| diff | | 4.979366 | .1046155 | | 4.688739 | 5.269993 |

```
    diff = mean(1) - mean(2)                                      t =  47.5968
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.99416

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 1.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.0000
```

## $Cam2^{C580Y}$ vs. $Cam2^{rev}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2D)

```
. ttest logCam2, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | 2.525393 | .1843995 | .3193893 | 1.731986 | 3.3188 |
| 2 | 3 | .867163 | .0518709 | .0898431 | .6439804 | 1.090346 |
| combined | 6 | 1.696278 | .3805589 | .9321752 | .7180201 | 2.674536 |
| diff | | 1.65823 | .1915562 | | .9325863 | 2.383874 |

```
    diff = mean(1) - mean(2)                                      t =  8.6566
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.31454

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9959         Pr(|T| > |t|) = 0.0082          Pr(T > t) = 0.0041
```

## CamWT vs. CamWT$^{C580Y}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2E)

```
. ttest logCamWT, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -.5885569 | .1905804 | .330095 | -1.408558 | .2314445 |
| 2 | 3 | 2.184728 | .0602016 | .1042722 | 1.925701 | 2.443754 |
| combined | 6 | .7980854 | .6265337 | 1.534688 | -.8124706 | 2.408642 |
| diff | | -2.773285 | .1998628 | | -3.510595 | -2.035975 |

```
    diff = mean(1) - mean(2)                                      t = -13.8759
Ho: diff = 0                     Satterthwaite's degrees of freedom =   2.3952

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0012         Pr(|T| > |t|) = 0.0024          Pr(T > t) = 0.9988
```

## Cam3.II$^{rev}$ vs. Cam3.II$^{C580Y}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2F)

```
. ttest logCam3IIC580Y, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -.5074539 | .3730999 | .646228 | -2.112773 | 1.097866 |
| 2 | 3 | 3.167337 | .1149281 | .1990612 | 2.672841 | 3.661832 |
| combined | 6 | 1.329941 | .8400516 | 2.057698 | -.8294799 | 3.489363 |
| diff | | -3.67479 | .3903998 | | -5.123775 | -2.225806 |

```
    diff = mean(1) - mean(2)                                      t =  -9.4129
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.37616

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0031         Pr(|T| > |t|) = 0.0062          Pr(T > t) = 0.9969
```

## V1/S vs. V1/S$^{R539T}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2G)

```
. ttest logV1S, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.208234 | .3612046 | .6256247 | -2.762372 | .3459035 |
| 2 | 4 | 2.972716 | .1999613 | .3999226 | 2.33635 | 3.609082 |
| combined | 7 | 1.18088 | .8622912 | 2.281408 | -.9290704 | 3.290831 |
| diff | | -4.18095 | .4128599 | | -5.447017 | -2.914884 |

```
    diff = mean(1) - mean(2)                                      t = -10.1268
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.21256

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0008         Pr(|T| > |t|) = 0.0015          Pr(T > t) = 0.9992
```

## F32-TEM vs. F32-TEM$^{M476I}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2H)

```
. ttest logF32TEM, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.609438 | 0 | 0 | -1.609438 | -1.609438 |
| 2 | 4 | .3982619 | .3075268 | .6150537 | -.5804257 | 1.37695 |
| combined | 7 | -.4621809 | .4376593 | 1.157938 | -1.533095 | .6087328 |
| diff | | -2.0077 | .3075268 | | -2.986388 | -1.029012 |

```
    diff = mean(1) - mean(2)                                      t =  -6.5285
Ho: diff = 0                     Satterthwaite's degrees of freedom =       3

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0037         Pr(|T| > |t|) = 0.0073          Pr(T > t) = 0.9963
```

## FCB vs. FCB$^{C580Y}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2I)

```
. ttest logFCB, by(group) unequal

Two-sample t test with unequal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |       3   -1.260332    .1874178    .3246172   -2.066726   -.4539377
       2 |       4    .5756699    .2217322    .4434645    -.129981    1.281321
---------+--------------------------------------------------------------------
combined |       7   -.2111879    .3957941    1.047173   -1.179661    .7572853
---------+--------------------------------------------------------------------
    diff |           -1.836002    .2903285               -2.582576   -1.089427
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                      t =  -6.3239
Ho: diff = 0                     Satterthwaite's degrees of freedom =  4.99418

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0007         Pr(|T| > |t|) = 0.0015          Pr(T > t) = 0.9993
```

## Dd2 vs. Dd2$^{Y493H}$ (groups 1 vs. 2 respectively; $p$<0.05; Fig. 2J)

```
. ttest logDd2Y493H, by(group) unequal

Two-sample t test with unequal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |       3   -1.304008    .3314896    .5741568   -2.730292    .1222767
       2 |       3    .4804795    .2148663    .3721593   -.4440154    1.404975
---------+--------------------------------------------------------------------
combined |       6   -.4117641    .4363832    1.068916   -1.533523    .7099947
---------+--------------------------------------------------------------------
    diff |           -1.784487    .3950352               -2.957299   -.6116756
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                      t =  -4.5173
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.42843

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0076         Pr(|T| > |t|) = 0.0152          Pr(T > t) = 0.9924
```

## Dd2 vs. Dd2$^{C580Y}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2J)

```
. ttest logDd2C580Y, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.304008 | .3314896 | .5741568 | -2.730292 | .1222767 |
| 2 | 3 | 1.39194 | .1087222 | .1883124 | .9241457 | 1.859734 |
| combined | 6 | .043966 | .622694 | 1.525282 | -1.55672 | 1.644652 |
| diff | | -2.695947 | .3488637 | | -3.970905 | -1.42099 |

diff = mean(1) - mean(2) t = -7.7278
Ho: diff = 0 Satterthwaite's degrees of freedom = 2.42536

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
Pr(T < t) = 0.0046 Pr(|T| > |t|) = 0.0092 Pr(T > t) = 0.9954

## Dd2 vs. Dd2$^{M476I}$ (groups 1 vs. 2 respectively; $p$<0.001; Fig. 2J)

```
. ttest logDd2M476I, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.304008 | .3314896 | .5741568 | -2.730292 | .1222767 |
| 2 | 4 | 2.162859 | .2838199 | .5676399 | 1.259617 | 3.0661 |
| combined | 7 | .6770588 | .7275241 | 1.924848 | -1.103129 | 2.457246 |
| diff | | -3.466866 | .4363933 | | -4.634165 | -2.299568 |

diff = mean(1) - mean(2) t = -7.9444
Ho: diff = 0 Satterthwaite's degrees of freedom = 4.42262

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
Pr(T < t) = 0.0004 Pr(|T| > |t|) = 0.0009 Pr(T > t) = 0.9996

## Dd2 vs. Dd2$^{R539T}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2J)

```
. ttest logDd2R539T, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.304008 | .3314896 | .5741568 | -2.730292 | .1222767 |
| 2 | 3 | 2.96476 | .0417134 | .0722497 | 2.785281 | 3.144238 |
| combined | 6 | .830376 | .966149 | 2.366572 | -1.653189 | 3.313941 |
| diff | | -4.268767 | .3341038 | | -5.664839 | -2.872696 |

```
    diff = mean(1) - mean(2)                                      t = -12.7768
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.06332

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0027         Pr(|T| > |t|) = 0.0054          Pr(T > t) = 0.9973
```

## Dd2 vs. Dd2$^{I543T}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2J)

```
. ttest logDd2I543T, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.304008 | .3314896 | .5741568 | -2.730292 | .1222767 |
| 2 | 3 | 3.353108 | .1395851 | .2417684 | 2.752522 | 3.953694 |
| combined | 6 | 1.02455 | 1.053713 | 2.581058 | -1.684104 | 3.733204 |
| diff | | -4.657116 | .3596795 | | -5.880846 | -3.433385 |

```
    diff = mean(1) - mean(2)                                      t = -12.9480
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.68763

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0008         Pr(|T| > |t|) = 0.0017          Pr(T > t) = 0.9992
```

## FCB$^{C580Y}$ vs. Dd2$^{C580Y}$ (groups 1 vs. 2 respectively; $p$<0.05; Fig. 2K)

```
. ttest logDd2C580Y, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 4 | .5756699 | .2217322 | .4434645 | -.129981 | 1.281321 |
| 2 | 3 | 1.39194 | .1087222 | .1883124 | .9241457 | 1.859734 |
| combined | 7 | .9254998 | .2071994 | .5481982 | .418501 | 1.432499 |
| diff | | -.8162698 | .2469528 | | -1.486439 | -.1461009 |

diff = mean(1) - mean(2) t = -3.3054
Ho: diff = 0 Satterthwaite's degrees of freedom = 4.24766

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
Pr(T < t) = 0.0136 Pr(|T| > |t|) = 0.0272 Pr(T > t) = 0.9864

## FCB$^{C580Y}$ vs. CamWT$^{C580Y}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2K)

```
. ttest logCamWTC580Y, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 4 | .5756699 | .2217322 | .4434645 | -.129981 | 1.281321 |
| 2 | 3 | 2.184728 | .0602016 | .1042722 | 1.925701 | 2.443754 |
| combined | 7 | 1.265266 | .3467581 | .9174356 | .4167797 | 2.113753 |
| diff | | -1.609058 | .2297595 | | -2.290976 | -.9271395 |

diff = mean(1) - mean(2) t = -7.0032
Ho: diff = 0 Satterthwaite's degrees of freedom = 3.43063

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
Pr(T < t) = 0.0019 Pr(|T| > |t|) = 0.0038 Pr(T > t) = 0.9981

## FCB$^{C580Y}$ vs. Cam2$^{C580Y}$ (groups 1 vs. 2 respectively; $p$<0.01; Fig. 2K)

```
. ttest logCam2C580Y, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 4 | .5756699 | .2217322 | .4434645 | -.129981 | 1.281321 |
| 2 | 3 | 2.525393 | .1843995 | .3193893 | 1.731986 | 3.3188 |
| combined | 7 | 1.411266 | .4172107 | 1.103836 | .3903876 | 2.432143 |
| diff | | -1.949723 | .2883892 | | -2.691124 | -1.208322 |

```
    diff = mean(1) - mean(2)                                      t =  -6.7607
Ho: diff = 0                     Satterthwaite's degrees of freedom =  4.99837

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0005         Pr(|T| > |t|) = 0.0011          Pr(T > t) = 0.9995
```

## FCB$^{C580Y}$ vs. Cam3.II$^{C580Y}$ (groups 1 vs. 2 respectively; $p$<0.001; Fig. 2K)

```
. ttest logCam3IIC580Y, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 4 | .5756699 | .2217322 | .4434645 | -.129981 | 1.281321 |
| 2 | 3 | 3.167337 | .1149281 | .1990612 | 2.672841 | 3.661832 |
| combined | 7 | 1.686384 | .5385969 | 1.424993 | .3684851 | 3.004283 |
| diff | | -2.591667 | .2497471 | | -3.263246 | -1.920088 |

```
    diff = mean(1) - mean(2)                                      t = -10.3772
Ho: diff = 0                     Satterthwaite's degrees of freedom =  4.35678

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0002         Pr(|T| > |t|) = 0.0003          Pr(T > t) = 0.9998
```

## Dd2 vs. $Dd2^{ctrl}$ (groups 1 vs. 2 respectively; $p$ = not significant; Fig. S2)

```
. ttest logDd2, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.304008 | .3314896 | .5741568 | -2.730292 | .1222767 |
| 2 | 3 | -.3955087 | .2486298 | .4306395 | -1.465276 | .6742591 |
| combined | 6 | -.8497582 | .2749708 | .6735382 | -1.556593 | -.1429232 |
| diff | | -.908499 | .4143695 | | -2.095421 | .2784229 |

```
    diff = mean(1) - mean(2)                                      t =  -2.1925
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.70929

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0494         Pr(|T| > |t|) = 0.0988          Pr(T > t) = 0.9506
```

## FCB vs. $FCB^{ctrl}$ (groups 1 vs. 2 respectively; $p$ = not significant; Fig. S2)

```
. ttest logFCB, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.260332 | .1874178 | .3246172 | -2.066726 | -.4539377 |
| 2 | 3 | -.8974407 | .3438403 | .5955488 | -2.376866 | .5819846 |
| combined | 6 | -1.078886 | .193015 | .4727884 | -1.575047 | -.5827252 |
| diff | | -.3628909 | .3916013 | | -1.588419 | .8626372 |

```
    diff = mean(1) - mean(2)                                      t =  -0.9267
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.09202

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2103         Pr(|T| > |t|) = 0.4206          Pr(T > t) = 0.7897
```

V1/S vs. V1/S$^{ctrl}$ (groups 1 vs. 2 respectively; $p$ = not significant; Fig. S2)

```
. ttest logV1S, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.208234 | .3612046 | .6256247 | -2.762372 | .3459035 |
| 2 | 3 | -1.735915 | .1444376 | .2501733 | -2.35738 | -1.11445 |
| combined | 6 | -1.472075 | .2102107 | .514909 | -2.012439 | -.931711 |
| diff | | .5276807 | .3890128 | | -.8171156 | 1.872477 |

```
    diff = mean(1) - mean(2)                                      t =   1.3565
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.62366

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8601         Pr(|T| > |t|) = 0.2799          Pr(T > t) = 0.1399
```

F32-ART vs. F32-ART $^{ctrl}$ (groups 1 vs. 2 respectively; $p$ = not significant; Fig. S2)

```
. ttest logF32ART, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | 1.54359 | .2493203 | .4318355 | .470851 | 2.616329 |
| 2 | 5 | 1.056817 | .2388532 | .5340919 | .3936547 | 1.71998 |
| combined | 8 | 1.239357 | .1869997 | .5289149 | .7971732 | 1.681541 |
| diff | | .4867725 | .3452701 | | -.3917757 | 1.365321 |

```
    diff = mean(1) - mean(2)                                      t =   1.4098
Ho: diff = 0                     Satterthwaite's degrees of freedom =  5.17592

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8921         Pr(|T| > |t|) = 0.2158          Pr(T > t) = 0.1079
```

## Dd2 comparison between IPC and CNRS (groups 1 vs. 2 respectively; $p$ = not significant; Fig. S3)

```
. ttest logDd2, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -1.304008 | .3314896 | .5741568 | -2.730292 | .1222767 |
| 2 | 2 | -.4315231 | .5493061 | .7768362 | -7.411119 | 6.548073 |
| combined | 5 | -.9550139 | .3298683 | .7376079 | -1.870875 | -.0391527 |
| diff | | -.8724846 | .6415782 | | -4.057465 | 2.312496 |

```
    diff = mean(1) - mean(2)                                      t =  -1.3599
Ho: diff = 0                     Satterthwaite's degrees of freedom =  1.74524

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1615         Pr(|T| > |t|) = 0.3230          Pr(T > t) = 0.8385
```

## FCB$^{ctrl}$ comparison between NIH and CNRS (groups 1 vs. 2 respectively; $p$ = not significant; Fig. S3)

```
. ttest logFCB, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | -.8974407 | .3438403 | .5955488 | -2.376866 | .5819846 |
| 2 | 3 | -1.260332 | .1874178 | .3246172 | -2.066726 | -.4539377 |
| combined | 6 | -1.078886 | .193015 | .4727884 | -1.575047 | -.5827252 |
| diff | | .3628909 | .3916013 | | -.8626372 | 1.588419 |

```
    diff = mean(1) - mean(2)                                      t =   0.9267
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.09202

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.7897         Pr(|T| > |t|) = 0.4206          Pr(T > t) = 0.2103
```

Cam5$^{I543T}$ comparison between NIH and IPC (groups 1 vs. 2 respectively; $p$ = not significant; Fig. S3)

```
. ttest logCam5, by(group) unequal
```

Two-sample t test with unequal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 1 | 3 | 3.763479 | .0753753 | .1305539 | 3.439165 | 4.087793 |
| 2 | 2 | 3.685584 | .0181838 | .0257158 | 3.454537 | 3.916632 |
| combined | 5 | 3.732321 | .0458427 | .1025074 | 3.605041 | 3.859601 |
| diff | | .077895 | .0775377 | | -.2254468 | .3812368 |

```
    diff = mean(1) - mean(2)                                      t =   1.0046
Ho: diff = 0                     Satterthwaite's degrees of freedom =   2.2245

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.7942         Pr(|T| > |t|) = 0.4116          Pr(T > t) = 0.2058
```

**References and Notes**


1. R. Feachem, O. Sabot, A new global malaria eradication strategy. *Lancet* **371**, 1633–1635 (2008). Medline doi:10.1016/S0140-6736(08)60424-9

2. N. J. White, S. Pukrittayakamee, T. T. Hien, M. A. Faiz, O. A. Mokuolu, A. M. Dondorp, Malaria. *Lancet* **383**, 723–735 (2014). Medline doi:10.1016/S0140-6736(13)60024-0

3. N. J. White, Delaying antimalarial drug resistance with combination chemotherapy. *Parassitologia* **41**, 301–308 (1999). Medline

4. R. T. Eastman, D. A. Fidock, Artemisinin-based combination therapies: A vital tool in efforts to eliminate malaria. *Nat. Rev. Microbiol.* **7**, 864–874 (2009). Medline

5. World Health Organization, World Malaria Report: 2013. WHO Press: Geneva, Switzerland. http://www.who.int/malaria/publications/world_malaria_report_2013/en/

6. A. M. Dondorp, F. Nosten, P. Yi, D. Das, A. P. Phyo, J. Tarning, K. M. Lwin, F. Ariey, W. Hanpithakpong, S. J. Lee, P. Ringwald, K. Silamut, M. Imwong, K. Chotivanich, P. Lim, T. Herdman, S. S. An, S. Yeung, P. Singhasivanon, N. P. Day, N. Lindegardh, D. Socheat, N. J. White, Artemisinin resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.* **361**, 455–467 (2009). Medline doi:10.1056/NEJMoa0808859

7. E. A. Ashley, M. Dhorda, R. M. Fairhurst, C. Amaratunga, P. Lim, S. Suon, S. Sreng, J. M. Anderson, S. Mao, B. Sam, C. Sopha, C. M. Chuor, C. Nguon, S. Sovannaroth, S. Pukrittayakamee, P. Jittamala, K. Chotivanich, K. Chutasmit, C. Suchatsoonthorn, R. Runcharoen, T. T. Hien, N. T. Thuy-Nhien, N. V. Thanh, N. H. Phu, Y. Htut, K. T. Han, K. H. Aye, O. A. Mokuolu, R. R. Olaosebikan, O. O. Folaranmi, M. Mayxay, M. Khanthavong, B. Hongvanthong, P. N. Newton, M. A. Onyamboko, C. I. Fanello, A. K. Tshefu, N. Mishra, N. Valecha, A. P. Phyo, F. Nosten, P. Yi, R. Tripura, S. Borrmann, M. Bashraheil, J. Peshu, M. A. Faiz, A. Ghose, M. A. Hossain, R. Samad, M. R. Rahman, M. M. Hasan, A. Islam, O. Miotto, R. Amato, B. MacInnis, J. Stalker, D. P. Kwiatkowski, Z. Bozdech, A. Jeeyapant, P. Y. Cheah, T. Sakulthaew, J. Chalk, B. Intharabut, K. Silamut, S. J. Lee, B. Vihokhern, C. Kunasol, M. Imwong, J. Tarning, W. J. Taylor, S. Yeung, C. J. Woodrow, J. A. Flegg, D. Das, J. Smith, M. Venkatesan, C. V. Plowe, K. Stepniewska, P. J. Guerin, A. M. Dondorp, N. P. Day, N. J. White; Tracking Resistance to Artemisinin Collaboration (TRAC), Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.* **371**, 411–423 (2014). Medline doi:10.1056/NEJMoa1314981

8. D. L. Saunders, P. Vanachayangkul, C. LonU.S. Army Military Malaria Research ProgramNational Center for Parasitology, Entomology, and Malaria Control (CNM)Royal Cambodian Armed Forces, Dihydroartemisinin-piperaquine failure in Cambodia. *N. Engl. J. Med.* **371**, 484–485 (2014). Medline doi:10.1056/NEJMc1403007

9. R. Leang, A. Barrette, D. M. Bouth, D. Menard, R. Abdur, S. Duong, P. Ringwald, Efficacy of dihydroartemisinin-piperaquine for treatment of uncomplicated *Plasmodium falciparum* and Plasmodium vivax in Cambodia, 2008 to 2010. *Antimicrob. Agents Chemother.* **57**, 818–826 (2013). Medline doi:10.1128/AAC.00686-12

10. C. Amaratunga, S. Sreng, S. Suon, E. S. Phelps, K. Stepniewska, P. Lim, C. Zhou, S. Mao, J. M. Anderson, N. Lindegardh, H. Jiang, J. Song, X. Z. Su, N. J. White, A. M. Dondorp, T. J. Anderson, M. P. Fay, J. Mu, S. Duong, R. M. Fairhurst, Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: A parasite clearance rate study. *Lancet Infect. Dis.* **12**, 851–858 (2012). Medline

11. J. A. Flegg, P. J. Guérin, F. Nosten, E. A. Ashley, A. P. Phyo, A. M. Dondorp, R. M. Fairhurst, D. Socheat, S. Borrmann, A. Björkman, A. Mårtensson, M. Mayxay, P. N. Newton, D. Bethell, Y. Se, H. Noedl, M. Diakite, A. A. Djimde, T. T. Hien, N. J. White, K. Stepniewska, Optimal sampling designs for estimation of *Plasmodium falciparum* clearance rates in patients treated with artemisinin derivatives. *Malar. J.* **12**, 411 (2013). Medline doi:10.1186/1475-2875-12-411

12. B. Witkowski, C. Amaratunga, N. Khim, S. Sreng, P. Chim, S. Kim, P. Lim, S. Mao, C. Sopha, B. Sam, J. M. Anderson, S. Duong, C. M. Chuor, W. R. Taylor, S. Suon, O. Mercereau-Puijalon, R. M. Fairhurst, D. Menard, Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: In-vitro and ex-vivo drug-response studies. *Lancet Infect. Dis.* **13**, 1043–1049 (2013). Medline doi:10.1016/S1473-3099(13)70252-4

13. F. Ariey, B. Witkowski, C. Amaratunga, J. Beghain, A. C. Langlois, N. Khim, S. Kim, V. Duru, C. Bouchier, L. Ma, P. Lim, R. Leang, S. Duong, S. Sreng, S. Suon, C. M. Chuor, D. M. Bout, S. Ménard, W. O. Rogers, B. Genton, T. Fandeur, O. Miotto, P. Ringwald, J. Le Bras, A. Berry, J. C. Barale, R. M. Fairhurst, F. Benoit-Vical, O. Mercereau-Puijalon, D. Ménard, A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature* **505**, 50–55 (2014). Medline doi:10.1038/nature12876

14. I. H. Cheeseman, B. A. Miller, S. Nair, S. Nkhoma, A. Tan, J. C. Tan, S. Al Saai, A. P. Phyo, C. L. Moo, K. M. Lwin, R. McGready, E. Ashley, M. Imwong, K. Stepniewska, P. Yi, A. M. Dondorp, M. Mayxay, P. N. Newton, N. J. White, F. Nosten, M. T. Ferdig, T. J. Anderson, A major genome region underlying artemisinin resistance in malaria. *Science* **336**, 79–82 (2012). Medline doi:10.1126/science.1215966

15. S. Takala-Harrison, T. G. Clark, C. G. Jacob, M. P. Cummings, O. Miotto, A. M. Dondorp, M. M. Fukuda, F. Nosten, H. Noedl, M. Imwong, D. Bethell, Y. Se, C. Lon, S. D. Tyner, D. L. Saunders, D. Socheat, F. Ariey, A. P. Phyo, P. Starzengruber, H. P. Fuehrer, P. Swoboda, K. Stepniewska, J. Flegg, C. Arze, G. C. Cerqueira, J. C. Silva, S. M. Ricklefs, S. F. Porcella, R. M. Stephens, M. Adams, L. J. Kenefic, S. Campino, S. Auburn, B. MacInnis, D. P. Kwiatkowski, X. Z. Su, N. J. White, P. Ringwald, C. V. Plowe, Genetic loci associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment in Southeast Asia. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 240–245 (2013). Medline doi:10.1073/pnas.1211205110

16. O. Miotto, J. Almagro-Garcia, M. Manske, B. Macinnis, S. Campino, K. A. Rockett, C. Amaratunga, P. Lim, S. Suon, S. Sreng, J. M. Anderson, S. Duong, C. Nguon, C. M. Chuor, D. Saunders, Y. Se, C. Lon, M. M. Fukuda, L. Amenga-Etego, A. V. Hodgson, V. Asoala, M. Imwong, S. Takala-Harrison, F. Nosten, X. Z. Su, P. Ringwald, F. Ariey, C. Dolecek, T. T. Hien, M. F. Boni, C. Q. Thai, A. Amambua-Ngwa, D. J. Conway, A. A. Djimdé, O. K. Doumbo, I. Zongo, J. B. Ouedraogo, D. Alcock, E. Drury, S. Auburn, O. Koch, M. Sanders, C. Hubbart, G. Maslen, V. Ruano-Rubio, D. Jyothi, A. Miles, J. O'Brien, C. Gamble, S. O. Oyola, J. C. Rayner, C. I. Newbold, M. Berriman, C. C. Spencer, G. McVean, N. P. Day, N. J. White, D. Bethell, A. M. Dondorp, C. V. Plowe, R. M. Fairhurst, D. P. Kwiatkowski, Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. *Nat. Genet.* **45**, 648–655 (2013). Medline doi:10.1038/ng.2624

17. J. Adams, R. Kelso, L. Cooley, The kelch repeat superfamily of proteins: Propellers of cell function. *Trends Cell Biol.* **10**, 17–24 (2000). Medline doi:10.1016/S0962-8924(99)01673-6

18. B. Witkowski, J. Lelièvre, M. J. Lopez Barragán, V. Laurent, X.-Z. Su, A. Berry, F. Benoit-Vical, Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *Antimicrob. Agents Chemother.* **54**, 1872–1877 (2010). Medline doi:10.1128/AAC.01636-09

19. C. Amaratunga, B. Witkowski, N. Khim, D. Menard, R. M. Fairhurst, Artemisinin resistance in *Plasmodium falciparum*. *Lancet Infect. Dis.* **14**, 449–450 (2014). Medline doi:10.1016/S1473-3099(14)70777-7

20. J. Straimer, M. C. Lee, A. H. Lee, B. Zeitler, A. E. Williams, J. R. Pearl, L. Zhang, E. J. Rebar, P. D. Gregory, M. Llinás, F. D. Urnov, D. A. Fidock, Site-specific genome editing in *Plasmodium falciparum* using engineered zinc-finger nucleases. *Nat. Methods* **9**, 993–998 (2012). Medline doi:10.1038/nmeth.2143

21. M. Ghorbal, M. Gorman, C. R. Macpherson, R. M. Martins, A. Scherf, J. J. Lopez-Rubio, Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. *Nat. Biotechnol.* **32**, 819–821 (2014). Medline doi:10.1038/nbt.2925

22. T. Ponnudurai, J. H. Meuwissen, A. D. Leeuwenberg, J. P. Verhave, A. H. Lensen, The production of mature gametocytes of *Plasmodium falciparum* in continuous cultures of different isolates infective to mosquitoes. *Trans. R. Soc. Trop. Med. Hyg.* **76**, 242–250 (1982). Medline doi:10.1016/0035-9203(82)90289-9

23. C. Amaratunga, B. Witkowski, D. Dek, V. Try, N. Khim, O. Miotto, D. Ménard, R. M. Fairhurst, *Plasmodium falciparum* founder populations in western Cambodia have reduced artemisinin sensitivity in vitro. *Antimicrob. Agents Chemother.* **58**, 4935–4937 (2014). Medline doi:10.1128/AAC.03055-14

24. S. Takala-Harrison *et al*., Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. *J. Infect. Dis.* 10.1093/infdis/jiu491 (2014).

25. M. D. Conrad, V. Bigira, J. Kapisi, M. Muhindo, M. R. Kamya, D. V. Havlir, G. Dorsey, P. J. Rosenthal, Polymorphisms in K13 and falcipain-2 associated with artemisinin resistance are not prevalent in *Plasmodium falciparum* isolated from Ugandan children. *PLOS ONE* **9**, e105690 (2014). Medline doi:10.1371/journal.pone.0105690

26. S. M. Taylor *et al*., Absence of putative artemisinin resistance mutations among Plasmodium falciparum in Sub-Saharan Africa: A molecular epidemiologic study. *J. Infect. Dis*. 10.1093/infdis/jiu467 (2014).

27. N. Klonis, D. J. Creek, L. Tilley, Iron and heme metabolism in *Plasmodium falciparum* and the mechanism of action of artemisinins. *Curr. Opin. Microbiol.* **16**, 722–727 (2013). Medline doi:10.1016/j.mib.2013.07.005

28. C. L. Hartwig, A. S. Rosenthal, J. D'Angelo, C. E. Griffin, G. H. Posner, R. A. Cooper, Accumulation of artemisinin trioxane derivatives within neutral lipids of *Plasmodium falciparum* malaria parasites is endoperoxide-dependent. *Biochem. Pharmacol.* **77**, 322–336 (2009). Medline doi:10.1016/j.bcp.2008.10.015

29. T. Antoine, N. Fisher, R. Amewu, P. M. O'Neill, S. A. Ward, G. A. Biagini, Rapid kill of malaria parasites by artemisinin and semi-synthetic endoperoxides involves ROS-dependent depolarization of the membrane potential. *J. Antimicrob. Chemother.* **69**, 1005–1016 (2014). Medline doi:10.1093/jac/dkt486

30. N. Klonis, M. P. Crespo-Ortiz, I. Bottova, N. Abu-Bakar, S. Kenny, P. J. Rosenthal, L. Tilley, Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 11405–11410 (2011). Medline doi:10.1073/pnas.1104063108

31. S. Saralamba, W. Pan-Ngum, R. J. Maude, S. J. Lee, J. Tarning, N. Lindegårdh, K. Chotivanich, F. Nosten, N. P. Day, D. Socheat, N. J. White, A. M. Dondorp, L. J. White, Intrahost modeling of

artemisinin resistance in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 397–402 (2011). Medline doi:10.1073/pnas.1006113108

32. Y. S. Keum, B. Y. Choi, Molecular and chemical regulation of the Keap1-Nrf2 signaling pathway. *Molecules* **19**, 10074–10089 (2014). Medline doi:10.3390/molecules190710074

33. F. D. Urnov, E. J. Rebar, M. C. Holmes, H. S. Zhang, P. D. Gregory, Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* **11**, 636–646 (2010). Medline doi:10.1038/nrg2842

34. M. C. S. Lee, P. A. Moura, E. A. Miller, D. A. Fidock, *Plasmodium falciparum* Sec24 marks transitional ER that exports a model cargo via a diacidic motif. *Mol. Microbiol.* **68**, 1535–1546 (2008). Medline doi:10.1111/j.1365-2958.2008.06250.x

35. D. A. Fidock, T. Nomura, T. E. Wellems, Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. *Mol. Pharmacol.* **54**, 1140–1147 (1998). Medline

36. E. H. Ekland, J. Schneider, D. A. Fidock, Identifying apicoplast-targeting antimalarials using high-throughput compatible approaches. *FASEB J.* **25**, 3583–3593 (2011). Medline doi:10.1096/fj.11-187401

## Annex 7. Ethic Committee Approval for K13 mutation study in Kupang

**MEDICAL AND HEALTH RESEARCH ETHICS COMMITTEE (MHREC)**
**FACULTY OF MEDICINE GADJAH MADA UNIVERSITY**
**– DR. SARDJITO GENERAL HOSPITAL**

### ETHICS COMMITTEE APPROVAL

Ref : KE/FK/ 222 /EC

| | | |
|---|---|---|
| Title of the Research Protocol | : | Is There Emergence of Act (Artemisinin based Combination Therapy) Resistance in Indonesia |
| Documents Approved | : | 1. Study Protocol versi 01 2015<br>2. Information for Subjects versi 01 2015<br>3. Informed consent form versi 01 2015 |
| Principle Investigator | : | Dr. dr. Mahardika Agus Wijayanti, DTM&H, M.Kes |
| Participating Investigator(s) | : | Dr. dr. Eti Nurwening Sholikhah, M.Kes |
| Date of Approval | : | 23 FEB 2015<br>(Valid for one year beginning from the date of approval) |
| Institution(s)/place(s) of research | : | Lab Pharmacology & Therapy UGM, Laboratoire de Chimie de Coordination du CNRS |

The Medical and Health Research Ethics Committee (MHREC) states that the above protocol meets the ethical principle outlined in the Declaration of Helsinki 2008 and therefore can be carried out.

The Medical and Health Research Ethics Committee (MHREC) has the right to monitor the research activities at any time.

The investigator(s) is/are obliged to submit:
□ Progress report as a continuing review : Annually
□ Report of any serious adverse events (SAE)
☑ Final report upon the completion of the study

Prof. Dr. dr. Sri Sutarni, Sp.S(K)
Vice Chairperson

dr. Madarina Julia, Sp.A(K).,MPH.,Ph.D
Secretary

Attachments:
□ Continuing review submission form (AF 4.3.01-014.2013-03)
□ Serious adverse events (SAE) report form (AF 6.1.01- 019.2013-03)